BOTANICALS AND CARDIOMETABOLIC RISK

Articles in this supplement were generated through research and scientific conferences organized by the NIH Center for the Study of Botanicals and Metabolic Syndrome

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Guest Editor

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We would like to thank the individuals at the National Center for Complementary and Alternative Medicine (NCCAM) and the Office of Dietary Supplements (ODS) at the National Institutes of Health (NIH) whose interest and help for this supplement made it possible. Articles in this supplement were generated through research and scientific conferences organized by the NIH-funded Botanical Research Center whose theme is “Botanicals and Metabolic Syndrome.” This Center is located at the Pennington Biomedical Research Center, Louisiana State University System, in Baton Rouge, LA, and in collaboration with The Biotech Center at Rutgers University, the LSU Agricultural Center, and the Center for Advanced Nutrition, Utah State University. The body of scientific work presented in this supplement was supported by NIH Grant P50AT002776-01 from the National Center for Complementary and Alternative Medicine and the Office of Dietary Supplements, which funds the Botanical Research Center.

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Botanicals and cardiometabolic risk: positioning science to address the hype

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Metabolic syndrome describes the human condition characterized by the presence of coexisting traditional risk factors for cardiovascular disease such as hypertension, dyslipidemia, glucose intolerance, and obesity, in addition to nontraditional cardiovascular risk factors such as inflammatory processes and abnormalities of the blood coagulation system. Although the specific etiology for metabolic syndrome is not known, insulin resistance—a clinical state in which a normal or elevated insulin level reflects an impaired biologic response—is present and is considered a key pathophysiological abnormality. As such, metabolic syndrome can be considered to be a prediabetic state and contributes greatly to increased morbidity and mortality in humans. As the prevalence is now reaching epidemic proportions worldwide, metabolic syndrome represents one of the most important public health problems facing our society today; and successful strategies are direly needed to intervene in its development. Although it is well established that caloric restriction and exercise greatly improve insulin resistance, the success of lifestyle intervention in humans over a long-term period is poor. Therefore, strategies to improve insulin resistance and components of the metabolic syndrome by pharmacologic approaches have remained as a major focus for drug development and are a primary and extremely viable option in clinical medicine. Unfortunately, recent data have questioned the safety of the current pharmacologic approaches to improve insulin resistance [1]. In this regard, alternative strategies, for example, nutritional supplementation with over-the-counter botanical agents, are extensively practiced by a large number of individuals with chronic diseases and are frequently undertaken without first informing the medical provider. However, considerable controversy has existed regarding the routine use of most supplements on human health. In part, the controversy stems from the paucity of scientifically controlled clinical studies that have evaluated the effectiveness of any individual bioactive. In addition, the precise cellular mechanism of action for most botanicals has not been elucidated.

In an effort to address the above concerns, in 1999, the Office of Dietary Supplements (ODS) and the National Center for Complementary and Alternative Medicine (NCCAM) at the National Institutes of Health (NIH) collaborated in the development and funding of a botanical research initiative with major research institutions in the United States [2]. The Botanical Research Centers Program (BRCP) is the most visible activity of the initiative; and the program is intended to advance the spectrum of botanical research activities ranging from plant identification to early-phase clinical studies, with preclinical research encouraged as the primary focus of center activities [2]. Currently, the NIH funds 6 Dietary Supplement Research Centers focused on botanicals [2,3]. Each center has a thematic focus with high potential for being translated into benefits for human health. At the 2007 Experimental Biology Annual Meeting, the investigators from all 6 centers presented a workshop sharing research strategies used by the BRCP scientists and described approaches for testing the efficacy and safety of botanicals, particularly those used in dietary supplements. The entire body of that work summarizing the workshop was recently published [4].

As it relates to the studies reported for this special supplement, the theme for the Botanical Research Center that represents collaboration between the Pennington Biomedical Research Center of the Louisiana State University System, and the Biotech Center at Rutgers University is “Botanicals and Metabolic Syndrome.” The overall goal of our center is to provide a comprehensive evaluation of botanicals in addressing the pathophysiologic...
mechanisms that lead to the development of insulin resistance and the metabolic syndrome. Attainment of our goal will not only allow for specific investigation into the underlying mechanisms of this condition, but will provide the necessary data for future clinical trials for botanicals designed to intervene in the process. Thus, the articles in this supplement result from work supported by the NIH BRCP and range from overviews of botanical preparation and appropriate animal models for study of botanicals to specific studies by which botanicals may enhance cellular mechanisms of insulin action.

One of the more exciting lines of investigation featured in this supplement is the role that botanicals may play in epigenetics. Epigenetics refers to heritable changes in gene expression that are not attributable to changes in DNA sequence and impacts many areas of applied and basic biology. Epigenetic changes are known to contribute to aging in addition to multiple disease states; and epigenetic defects are thought to be more easily reversible (when compared with genetic defects) and, as such, have inspired efforts to identify novel compounds that correct epimutations or prevent progression to the disease state. Thus, it is exciting that a manuscript published in this supplement appears to be one of the first to report that botanical sources may be a rich source of agents that can potentially modulate the epigenome and related pathways and can potentially be useful in attenuating the progression of many factors related to the development of metabolic syndrome.

This special supplement of *Metabolism* would not have been possible without the funding of the BRCP by the NIH. Clearly, the time has come to provide the required science to either support a botanical for use in human health based on its effectiveness or to discourage use of a specific botanical based on no effect or even adverse effects. We hope you enjoy reading this supplement because the articles reported represent the rigorous and careful science needed for the study of botanicals.

Acknowledgment/Conflict of Interest

Supported by NIH Grant P50AT002776-01 from the National Center for Complementary and Alternative Medicine (NCCAM) and the Office of Dietary Supplements (ODS), which funds the Botanical Research Center of Pennington Biomedical Research Center and The Biotech Center of Rutgers University.

References

A natural history of botanical therapeutics
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Abstract

Plants have been used as a source of medicine throughout history and continue to serve as the basis for many pharmaceuticals used today. Although the modern pharmaceutical industry was born from botanical medicine, synthetic approaches to drug discovery have become standard. However, this modern approach has led to a decline in new drug development in recent years and a growing market for botanical therapeutics that are currently available as dietary supplements, drugs, or botanical drugs. Most botanical therapeutics are derived from medicinal plants that have been cultivated for increased yields of bioactive components. The phytochemical composition of many plants has changed over time, with domestication of agricultural crops resulting in the enhanced content of some bioactive compounds and diminished content of others. Plants continue to serve as a valuable source of therapeutic compounds because of their vast biosynthetic capacity. A primary advantage of botanicals is their complex composition consisting of collections of related compounds having multiple activities that interact for a greater total activity.

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1. Natural products and drug discovery

Historically, natural products have provided an endless source of medicine. Plant-derived products have dominated the human pharmacopoeia for thousands of years almost unchallenged [1]. In 1897, Arthur Eichengrün and Felix Hoffmann, working at Friedrich Bayer, created the first synthetic drug, aspirin. Aspirin (acetylsalicylic acid) was synthesized from salicylic acid, an active ingredient of analgesic herbal remedies. This accomplishment ushered in an era dominated by the pharmaceutical industry. In 1928, penicillin was discovered by Alexander Fleming, adding microbes as important sources of novel drugs. The role of plant-derived natural products in drug discovery has recently been diminished by the advent of structure activity–guided organic synthesis, combinatorial chemistry, and computational (in silico) drug design.

Despite drug discovery technology diversification and reduced funding for natural product–based drug discovery, natural products from plants and other biological sources remain an undiminished source of new pharmaceuticals. Industrial funding for natural product–based drug discovery has been declining from 1984 to 2003, yet the percentage of natural product–derived small molecule patents has remained relatively unchanged [2]. A comprehensive review of human drugs introduced since 1981 suggests that, of 847 small molecule–based drugs, 43 were natural products, 232 were derived from natural products (usually semisynthetically), and 572 were synthetic molecules. However, 262 of the 572 synthetic molecules had a natural product–inspired pharmacophore or could be considered natural product analogs [3]. Natural products continue to make the most dramatic impact in the area of cancer. From 155 anticancer drugs developed since the 1940s, only 27% could not be traced to natural products, with 47% being either a natural product or a direct derivation thereof. Only one drug, the anticancer compound sorafenib, could be traced to completely de novo combinatorial chemistry [3]. The above analysis did not include biologics and vaccines, which are derived from nature by definition.
2. Current categories of botanical products in the United States

The use of botanicals for improving human health has evolved independently in different regions of the world. The production, use, attitude, and regulatory aspects of botanicals continue to vary globally. In the United States, botanicals are categorized based on intended use, safety, regulatory status, and degree of characterization. The regulatory aspects of botanical products are an important issue when considering standardization and quality assessment because the regulations dictate some degree of the process. The basic regulatory categories are as follows:

Dietary Supplements, also commonly known as nutraceuticals, are products consisting of dietary components that are intended to supplement the diet and usually consist of vitamins, minerals, botanicals, and others. Dietary supplements are regulated by the Food and Drug Administration (FDA) under the Dietary Health and Education Act of 1994 (http://www.cfsan.fda.gov/~dms/lab-qhc.html), which makes the manufacturer responsible for ensuring the safety of the products but places the burden of proof upon the FDA for enforcement. This creates an unregulated environment where marketing powers retain control. Unless a dietary supplement contains a new ingredient, there is not even a mandate to register the product.

Drugs can be prescription drugs or over-the-counter drugs. These products require the most rigorous testing including 3 distinct phases of clinical testing to ensure safety and efficacy and close scrutiny by the FDA. Although most early pharmaceutical products were botanical preparations and at least 25% of the pharmaceuticals used today are based on plant-derived products [10], only pure compounds isolated from plants and subjected to the same rigorous synthetic pharmaceutical can be conventional drugs. Botanically derived pharmaceuticals that are currently being used today include taxol and morphine [4].

Botanical Drugs are complex extracts from a plant to be used for the treatment of disease. The guidelines for this relatively new regulatory category were released in 2004 (http://www.fda.gov/cder/Guidance/4592fnl.htm). Botanical drugs are clinically evaluated for safety and efficacy just as conventional drugs, but the process for botanical drugs can be expedited because of the history of safe human use. Botanical drugs are highly but not completely characterized and are produced under the same strictly regulated conditions as conventional pharmaceuticals. Botanical drugs, such as senna and psyllium, can be marketed and sold under the FDA’s over-the-counter drug monograph system [11].

3. Plant domestication and secondary metabolites

Recent archeological records suggest that modern agriculture started in the Near East 10 000 to 11 000 years ago with the domestication of figs, cereals, and legumes [12,13]. At that time, early Neolithic farmers maintained a subsistence strategy, collecting wild plants for food and medicine while simultaneously domesticating early crops. This point in time marked the beginning of the divergence between medicinal plants and food plants. Centuries of plant domestication improved flavor, color, yield, uniformity, disease and pest resistance, reproductive fitness, and postharvest integrity of crops but has reduced pharmacologically active compounds from major crops to levels where average daily consumption cannot produce a measurable pharmacological effect. The pharmacological side effects of food, frequently residing in poorly palatable compounds, were not likely to be preserved or even considered advantageous by our ancestors. As a result, conventional plant breeding has often reduced the content of bioactive compounds in crops (Table 1).

For example, a wild tomato (Lycopersicon esculentum var cerasiforme) indigenous to Peru produces fruit with very high levels of the bitter glycoalkaloid tomatine (500-5000 mg/kg dry weight) [26]. Tomatine plays a role in pest and disease resistance and also has multiple pharmacological effects in humans including cholesterol-lowering, immunomodulatory,
and cardiotoxic [27,28]. Its ability to inhibit acetylcholines-
terase may be responsible for its potentially toxic effects [16].
Not surprisingly, tomatine is considerably lower in sweet
fruited tomato cultivars (∼30 mg/kg) [26], reducing the bitter
flavor but also reducing potential health benefits. Wild potato
species also contain considerably higher amounts of
glycoalkaloids than modern cultivars [29].

Wild bean species (Phaseolus vulgaris) contain many
secondary metabolites that are found in lower levels in
cultivated species including trypsin inhibitors, tannins, and
lectins [30]. These phytochemicals have been called anti-
nutritional because they may interfere with protein diges-
tion, although they have potential human health benefits as a
therapy for cancer, heart disease, and diabetes [31].

Contrary to the trend of reducing bioactives through
centuries of plant breeding, some bioactive compounds have
been fortuitously enhanced in modern food crops because
they impart desirable attributes like color or flavor (Table 2).
Examples include pigments such as carotenoids [43,44] and
flavonoids [45,46], aromatic constituents of volatile oils like
menthol [47,48], and other flavor constituents including
gingerols [49] and capsaicin [50,51].

Modern agriculture has also improved various medicinal
plants through years of selective breeding for bioactive
compounds. For example, foxglove (Digitalis purpurea) produces
digitoxin and digoxin, cardiac glycosides used to
treat congestive heart failure. Modern agriculture has created
uniform cultivars with high digoxin content [52]. Many
common cultivated plants are also the source of compounds
used as building blocks in the semisynthesis of pharmaceu-
ticals. A number of useful phytochemicals are extracted from
soybean (Glycine max) including the sterols stigmasterol,
sitosterol, and campesterol [53]. Sitosterol and campesterol
are esterified into plant stanol and sterol esters, both of which
have been shown to lower serum cholesterol [54]. Stigmas-
terol and sitosterol are used in the semisynthesis of
pharmaceutical steroids including progestagens, androgens,
and corticosteroids [53,55]. Diosgenin, a structurally related
steroid from Mexican yams (Dioscorea spp), is also used in
the semisynthesis of pharmaceutical steroids [53]. The
opium poppy (Papaver somniferum) produces morphinan
alkaloids including morphine, codeine, thebaine, papaverine,
and noscapine [56]. Opioid semisynthetic drugs include
dihydromorphine, fentanyl, and oxycodone [53]. Opioids are
widely used as powerful analgesics, cough suppressants,
and sedatives.

4. The power of biochemical potentiation

A recent review article defined potentiation as positive
interactions that intensify the potency of a bioactive product
[57]. Additive and synergistic effects are subsets of
potentiation, where 2 or more compounds in a mixture

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Table 1
Bioactive compounds that have been reduced in modern food crops

<table>
<thead>
<tr>
<th>Crop</th>
<th>Latin name</th>
<th>Chemical class</th>
<th>Examples</th>
<th>Targets</th>
<th>Toxicity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cassava</td>
<td>Manihot esculenta</td>
<td>Cynogenic glycosides</td>
<td>Linamarin, lotaustralin</td>
<td>Cancer</td>
<td>Block cellular respiration</td>
<td>[14]</td>
</tr>
<tr>
<td>Celer</td>
<td>Apium graveolens</td>
<td>Panauricoumarins</td>
<td>Psoralen, xanthotoxin, bergapten</td>
<td>Anticoagulant</td>
<td>Photosensitivity</td>
<td>[15,16]</td>
</tr>
<tr>
<td>Broccoli</td>
<td>Brassica napus</td>
<td>Glucosinolates</td>
<td>Sulforaphane</td>
<td>Cancer</td>
<td>Goitrogenic</td>
<td>[17,18]</td>
</tr>
<tr>
<td>Tomato</td>
<td>L esculentum</td>
<td>Glycoalkaloids</td>
<td>α-Chaconine, α-solaneine</td>
<td>Cancer</td>
<td>Neurotoxic</td>
<td>[19,29]</td>
</tr>
<tr>
<td>Common bean</td>
<td>P vulgaris</td>
<td>Glycoproteins</td>
<td>Lecitins</td>
<td>Cancer, HIV</td>
<td>Agglutination</td>
<td>[30,31]</td>
</tr>
<tr>
<td>Cotton</td>
<td>Gossypium spp</td>
<td>Phenolic sesquiterpenes</td>
<td>Gossypol</td>
<td>Cancer, male</td>
<td>contraceptive</td>
<td>[20,21]</td>
</tr>
<tr>
<td>Lettuce</td>
<td>Lactuca sativa</td>
<td>Sesquiterpene lactones</td>
<td>Lactucin, deoxyxactucin, lactucopicerin</td>
<td>Inflammation, malaria</td>
<td>Allergenic</td>
<td>[22,23]</td>
</tr>
</tbody>
</table>

Table 2
Bioactive compounds that have been increased in modern food crops

<table>
<thead>
<tr>
<th>Plant</th>
<th>Latin name</th>
<th>Chemical</th>
<th>Targets</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peppers</td>
<td>Capsicum spp</td>
<td>Capsaicin</td>
<td>Pain</td>
<td>[50,51]</td>
</tr>
<tr>
<td>Grapes</td>
<td>Vitis spp</td>
<td>Flavonoids</td>
<td>Cardiovascular disease</td>
<td>[32,45]</td>
</tr>
<tr>
<td>Peppermint</td>
<td>Mentha x piperita</td>
<td>Menthol</td>
<td>Decongestant</td>
<td>[47,48]</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>L esculentum</td>
<td>Lycopenes</td>
<td>Prostate cancer</td>
<td>[33,34]</td>
</tr>
<tr>
<td>Hops</td>
<td>Humulus lupulus</td>
<td>Humulene</td>
<td>Anti-inflammatory</td>
<td>[35,36]</td>
</tr>
<tr>
<td>Turmeric</td>
<td>Curcuma domestica</td>
<td>Curcumin</td>
<td>Inflammation, cancer</td>
<td>[37,49]</td>
</tr>
<tr>
<td>Ginger</td>
<td>Zingiberis rhizoma</td>
<td>Gingerols</td>
<td>Antieptic</td>
<td>[38,39]</td>
</tr>
<tr>
<td>Saffron</td>
<td>Crocus sativus</td>
<td>Carotenoids</td>
<td>Cancer</td>
<td>[40,41]</td>
</tr>
<tr>
<td>Apricots</td>
<td>Prunus armeniaca</td>
<td>Carotenoids</td>
<td>Cancer</td>
<td>[42,43]</td>
</tr>
</tbody>
</table>
interact to provide a combined effect that is equal to the sum of the effects of the individual components (additive) or where combinations of bioactive substances exert effects that are greater than the sum of individual components (synergistic). Potentiation can exist between 2 phytochemicals in a single plant extract, 2 phytochemicals from 2 different plant extracts, or between a phytochemical and synthetic drug. To validate this phenomenon, the bioactive

Table 3
Bioactive compounds isolated from an extract of A dracunculus L by activity-guided fractionation that inhibit the aldose reductase enzyme, protein tyrosine phosphatase 1B activity, and expression, or phosphoenolpyruvate carboxykinase overexpression [59-61]

<table>
<thead>
<tr>
<th>Isolated compounds</th>
<th>ALR2</th>
<th>PTP-1B</th>
<th>PEPCK</th>
</tr>
</thead>
<tbody>
<tr>
<td>4,5-Di-O-caffeoylquinic acid&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>Active</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Davidigenin&lt;sup&gt;abe&lt;/sup&gt;</td>
<td>Active</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6-Demethoxydihydrochalcone&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>Active</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2',4'-Dihydroxy-4-methoxydihydrochalcone&lt;sup&gt;abc,def&lt;/sup&gt;</td>
<td>Active</td>
<td>Active</td>
<td>Active</td>
</tr>
<tr>
<td>2',4-Dihydroxy-4'-methoxydihydrochalcone&lt;sup&gt;b,de&lt;/sup&gt;</td>
<td>Active</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sakuranetin&lt;sup&gt;bc,fg&lt;/sup&gt;</td>
<td>Active</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**Notes:**

- **ALR2** indicates aldose reductase; **PTP-1B**, protein tyrosine phosphatase 1B; **PEPCK**, phosphoenolpyruvate carboxykinase.
- <sup>a</sup> Confirmed with nuclear magnetic resonance.
- <sup>b</sup> New compound to A dracunculus.
- <sup>c</sup> Activity reported for the first time.
- <sup>d</sup> Dihydrochalcone.
- <sup>e</sup> New compound to genus Artemisia.
- <sup>f</sup> First report as a constituent of plants.
- <sup>g</sup> Flavonoid.

Table 4
Examples of potentiating interactions between various natural products with other natural products or drugs in the fields of cancer and antibiotic research

<table>
<thead>
<tr>
<th>Natural products</th>
<th>Drug/natural product</th>
<th>Target</th>
<th>Summary</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cancer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apple extracts&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Vitamin C</td>
<td>Liver cancer</td>
<td>Apple extracts, containing vitamin C, had greater antioxidant activity and reduced in vitro tumor proliferation greater than vitamin C alone.</td>
<td>[65]</td>
</tr>
<tr>
<td>Soy extract, genistein</td>
<td>Tamoxifen</td>
<td>Breast cancer</td>
<td>Combination of tamoxifen with genistein or soy extract had synergistic effects on delaying the growth of MCF-7 tumors in mice.</td>
<td>[66]</td>
</tr>
<tr>
<td>Tomato powder&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Lycopene</td>
<td>Prostate cancer</td>
<td>Tomato powder inhibited prostate cancer more than pure lycopene in NMU rats, suggesting that tomato powder contains compounds in addition to lycopene that modify prostate carcinogenesis.</td>
<td>[67]</td>
</tr>
<tr>
<td>Tomato powder, broccoli powder, combination of tomato and broccoli powders</td>
<td>Lycopene, finasteride</td>
<td>Prostate cancer</td>
<td>Tomato powder, broccoli powder, and combination treatments all significantly reduced Dunning rat prostate tumor size more than finasteride or lycopene. The tomato/broccoli combination treatment had the greatest antitumor effect.</td>
<td>[33]</td>
</tr>
<tr>
<td><strong>Antibacterial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acorus calamus, Hemidesmus indicus, Holarrhena antidysenterica, Plumbago zeylanica extracts and fractions</td>
<td>Ceftazidime, cefuroxime, chloramphenicol, ciprofloxacin, tetracycline</td>
<td>MRSA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Extracts and fractions from 4 plants demonstrated synergistic antibiotic activity against various strains of MRSA when used in combination with each other and in combination with synthetic antibiotics.</td>
<td>[68]</td>
</tr>
<tr>
<td>Tetracycline, ciprofloxacin</td>
<td>ESβL-producing E coli</td>
<td>ESβL-producing E coli</td>
<td>Extracts and fractions from 4 plants demonstrated synergistic antibiotic activity against ESβL-producing E coli when combined with tetracycline or ciprofloxacin.</td>
<td>[70]</td>
</tr>
<tr>
<td>Bidwillon B</td>
<td>Mupirocin</td>
<td>MRSA</td>
<td>When bidwillon B and mupirocin were combined, synergistic effects were observed for 11 strains of MRSA.</td>
<td>[72]</td>
</tr>
<tr>
<td>Camellia sinensis, Lawsonia inermis, Punica granatum, Terminalia belerica, Terminalia chebula extracts</td>
<td>Tetracycline, ampicillin</td>
<td>MRSA</td>
<td>The C sinensis extract showed synergism with ampicillin; L inermis, P granatum, T belerica, and T chebula extracts showed synergism with tetracycline.</td>
<td>[69]</td>
</tr>
</tbody>
</table>

**Notes:**

- MRSA indicates methicillin-resistant *S aureus*; ESβL, extendend spectrum beta-lactomases; NMU, N-methyl-N-nitrosourea.
- <sup>a</sup> Apple extracts contain naturally occurring vitamin C.
- <sup>b</sup> Tomato powder contains naturally occurring lycopene.
phytochemical(s) in a mixture must first be identified and isolated. Afterward, plant extracts or mixtures of phytochemicals must be tested side by side with the single bioactive compounds to see which one has greater bioactivity. Only then can clear conclusions be made whether or not a mixture of compounds actually intensifies the potency of a single bioactive product. A good example of the multicomponent nature of botanicals is illustrated with an extract from Artemisia dracunculus L that is being researched as a botanical therapeutic for diabetes and metabolic syndrome. The extract decreases blood glucose in hyperglycemic animal models of diabetes and seems to enhance insulin sensitivity as a mode of action [58]. Based on 3 of the diabetes-related activities identified for the extract, together with activity-guided fractionation, 6 active compounds were isolated and identified (Table 3). Therefore, the activity of the total extract is the combined result of at least 6 different compounds and at least 3 different activities. The precise nature of their interaction has not yet been defined.

In the field of cancer research, phytochemicals have been shown to affect various parts of signal transduction pathways including gene expression, cell cycle progression, proliferation, cell mortality, metabolism, and apoptosis [62]. Combination chemotherapy has been the mainstay of cancer treatment for 40 years [63]. It is therefore reasonable to assume that a mixture of compounds (phytochemical or synthetic) would have greater bioactivity than a single compound because a mixture of bioactive compounds has the ability to affect multiple targets [62,64]. Studies have documented synergistic anticancer effects of phytochemicals including quercetin, catechins, resveratrol, and curcumin with various cancer drugs and/or other phytochemicals [62]. A few other examples of synergistic anticancer activity are shown in Table 4. In addition, natural products have been shown to overcome multiple drug resistance in tumors when used in combination with other natural products or drugs [62]. Similar observations have been made in the field of antibiotic research (Table 4). A number of plant extracts and natural products have been shown to work synergistically with existing antibiotics, restoring antibiotic activity against resistant strains of Staphylococcus aureus (methicillin resistant), Escherichia coli, and Shigella [70-72].

5. Conclusions

Plants must maintain and protect themselves through diverse arrays of complex natural products that they make from the inorganic components of air, soil, and water because they lack the flight response. Remarkably, the oldest known living eukaryotic organism, turning 4772 years old in 2007, is a specimen of a bristlecone pine, Pinus longaeva, growing in the White Mountains of Inyo County, California [73]. Many other plants can live hundreds of years without succumbing to diseases or predation. It should come to no surprise that some of the compounds that have enabled plants to survive may also be used to maintain the health and well-being of humans.

Acknowledgment/Conflict of Interest

Research supported by NIH Grant P50 AT002776-01 from the National Center for Complementary and Alternative Medicine (NCCAM) and Office of Dietary Supplements (ODS) which funds the Botanical Research Center; also supported by Fogarty International Center of the National Institutes of Health under U01 TW006674 for the International Cooperative Biodiversity Groups; and Rutgers University. Support also received from Phytomedics, Inc (Jamesburg, NJ). David Ribnicky, Alexander Poulev, and Ilya Raskin serve as consultants for Phytomedics.

References


Strategies for assessment of botanical action on metabolic syndrome in the mouse and evidence for a genotype-specific effect of Russian tarragon in the regulation of insulin sensitivity

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Abstract

Published reports of botanical action are often hampered by the lack of generalized systematic approaches or by the failure to explore mechanisms that could confirm and extend the reported observations. Choice of mouse or rat housing conditions (singly or group housed) and imposed stress during handling procedures are often variable and can contribute significantly to differences in baseline phenotypes measured across studies. Differences can also be observed in the role of the extract in either the treatment of the metabolic syndrome or roles in the regulation of the emergence of metabolic syndrome. The choice of diet used can also vary between the different studies, and diet-botanical interactions must be considered. This minireview highlights the strategies being pursued by the Botanical Research Center Animal Research Core to evaluate the in vivo phenotypes of several botanical extracts during long-term feeding studies. We describe a phenotyping strategy that promotes a more rigorous interpretation of botanical action and can suggest or eliminate possible mechanisms that may be involved. We discuss the importance of selecting the mouse model, as background strain can significantly alter the underlying susceptibilities to the various components of metabolic syndrome. Finally, we present data suggesting that one of the major botanical extracts being studied, an extract of Russian tarragon, may manifest a mouse strain genotype–specific insulin-sensitizing phenotype.

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1. Introduction

Obesity and type 2 diabetes mellitus are both components of a generalized disorder termed metabolic syndrome [1]. Accumulation of excess adipose tissue, a hallmark of obesity, leads to insulin resistance that precedes the development of type 2 diabetes mellitus. Other metabolic syndrome–associated comorbidities include dyslipidemia and hypertension. Individually, these components of metabolic syndrome are themselves considered as complex diseases, regulated by a poorly understood interaction of genetics, diet, and physical activity. The public health initiatives adopted in the United States and elsewhere have typically focused on reductions in food intake and increases in physical activity. The continued increase in the incidence of these diseases, however, in the developed countries, coupled with the emerging epidemic in developing nations and its increasing prevalence in children, suggests that other strategies are essential.

The identification and characterization of botanical extracts that can modulate the development of chronic diseases of obesity and type 2 diabetes mellitus are the major focus of the Botanical Research Center (BRC) based jointly at the Pennington Biomedical Research Center in Baton Rouge, LA, and Rutgers University, NJ. This minireview discusses the contribution of the Animal Research Core of the Botanical Center and the use of differing murine genetic models of metabolic syndrome to help define the mechanism of action of botanicals.

2. Rationale for the use of the mouse as a model for human metabolic syndrome

Despite more than 65 million years since the divergence of primate and rodent phylogenetic lineages, comparative analysis of the human and mouse genomes reveals that 99%
of the genes associated with disease are evolutionarily conserved. Several attributes make the mouse an ideal experimental model for biomedical research: chronic diseases such as obesity, diabetes, cancer, autoimmunity, and muscular dystrophies seen in humans can and do occur to varying extents in different inbred mouse strains; they are easy and cost-effective to breed and maintain; a large number of genetically distinct inbred mouse strains exist; and the mouse genome is easily manipulated to generate transgenic and gene-deficient mutant mice. In addition, the genome of the commonly used C57BL/6J (B6) mouse has been completely sequenced (www.ensembl.org), making it easier to characterize the underlying genetic influences on phenotypic diversity.

3. Common mouse models for metabolic syndrome

Murine polygenic and monogenic models of the metabolic syndrome have been used to differing extents to characterize the effects of botanical extracts in the prevention and treatment of obesity and/or diabetes. Given that human obesity is predominantly polygenic in nature and overall genetic susceptibility can be modulated by energy-dense high-fat diets, the C57BL/6J (B6) high-fat dietary-induced obesity model is commonly used as a surrogate for the human disease. Male B6 mice, when fed a high-fat or high-fat + high-sucrose diet, rapidly develop obesity and manifest increased insulin resistance, hyperglycemia, dyslipidemia, and hypertension, all aspects of the metabolic syndrome [2,3]. The extent to which this mouse strain demonstrates a diabetes phenotype however is limited. The diabetes phenotype appears transient in nature and can resolve.

Several commonly used inbred mouse strains are resistant to high-fat dietary-induced obesity [4,5]. For example, the 129-related mouse strains (embryonic stem cells of which are used in gene targeting experiments) do not demonstrate obesity when fed a high-fat diet. Mechanisms that regulate increased energy expenditure are more effectively induced in this mouse strain relative to the B6 inbred strain [6].

Another commonly used mouse obesity and diabetes model is the leptin-deficient obesity mouse mutant homozygous for the lep<sup>ob</sup> allele. The leptin receptor (lep<sup>ob</sup>)–deficient mouse mutant also possesses a similar phenotype. Leptin is an important hormone, predominantly secreted by fat cells that interact with the leptin receptor isoforms expressed by neurons located in the hypothalamus. Changes in the level of circulating leptin alter feeding behavior, metabolism, and endocrine function [7]. Both male and female lep<sup>ob</sup> mutants are obese and demonstrate hyperglycemia, glucose intolerance, elevated plasma insulin, subfertility, impaired wound healing, and increases in hormone production from both pituitary and adrenal glands. They are also hypometabolic and hypothermic, necessitating a higher temperature (>25°C) in the animal rooms to minimize thermogenic stress. It is quite common to see an incorrect use of murine genetic nomenclature in the botanical-related literature when describing leptin-deficient mice. Instead of being described as “B6-ob or ob/ob mice,” the correct nomenclature of this mutant congenic strain when the mutation is on the B6 genetic background is C57BL/6.V-lep<sup>ob</sup> (abbreviated as B6.V-lep<sup>ob</sup>). Genetic background plays an important role in regulating the diabetic phenotype in leptin-deficient mice. The B6.V-lep<sup>ob</sup> strain, although demonstrating significant obesity, may not be the ideal choice to use in studies requiring significantly greater insulin resistance and type 2 diabetes mellitus. When the lep<sup>ob</sup> mutation is homozygous on the closely related C57BL/Ks genetic background, the congenic C57BL/Ks.V-lep<sup>ob</sup> mice are more severely diabetic, with regression of pancreatic islets and early adult lethality, than B6.V-lep<sup>ob</sup> mutants [8]. B6.V-lep<sup>ob</sup> mice live longer, in large part because they demonstrate only a transient hyperglycemia that resolves at around 14 to 15 weeks of age [9]. BALB/cJ congenic mice, when homozygous for the lep<sup>ob</sup> mutation, are leaner than B6.V-lep<sup>ob</sup> mutant mice and hence more fertile; but they demonstrate significantly greater diabetes [10]. Conversely, BTBR mice homozygous for Lep<sup>ob</sup> are more obese than B6.V-Lep<sup>ob</sup> mutant mice [11,12]. Thus, the same mutation on differing genetic backgrounds alters the degree of obesity, fertility, and diabetes. A potential drawback to the use of Lep<sup>ob</sup> mutant mice as a model for metabolic syndrome is that the data generated must be interpreted in light of a leptin-deficient environment. Although leptin-deficient humans have been identified in the population, the incidence of this mutation is quite rare. Most human obesity and diabetes are attributable to a more complex polygenic origin.

The KK.Cg-A<sup>y</sup> mutant mouse, also used as a model for metabolic syndrome, offers advantages over the use of the leptin-deficient mouse in that the development of obesity, insulin resistance, and diabetes in this model is polygenic and the mutant mice possess intact leptin and leptin receptor genes. The A<sup>y</sup>, or yellow mutation, contains a 170–base pair deletion at the nonagouti (a) locus that extends into the upstream Raly gene [13]. As a result, expression of agouti is under the control of the Raly gene promoter. Thus, agouti is ectopically expressed in all tissues instead of its more limited expression profile in the skin. Cross talk between agouti and specific melanocortin receptors in the hypothalamus of A<sup>y</sup>/+ heterozygous mice results in hyperphagia leading to an age-dependent metabolic syndrome phenotype [14-16]. Mice homozygous for A<sup>y</sup>/A<sup>y</sup> die in utero, presumably because of loss of Raly gene expression. Thus, genotyping of progeny derived from +/+ × A<sup>y</sup>/+ mating schemes is not required; yellow-colored A<sup>y</sup>/+ mice are easily distinguished from +/+ littermates. However, as found for different Lep<sup>ob</sup> mouse mutant strains, genetic background effects can significantly modify the metabolic syndrome phenotype in A<sup>y</sup>/+ mouse mutants.

Fig. 1 describes the effects of the A<sup>y</sup> mutation when congenic on the B6 and KK inbred mouse strain genetic
backgrounds. B6.Cg-\(A^{+/+}\) mice demonstrate a significant increase in body weight and adiposity, relative to the parental B6 mice, because of accumulation of fat mass that begins shortly after 7 weeks of age (Fig. 1, \(t = 0\)) and continues to increase for the next 3 months. In contrast, the parental KK/HJ (KK) and congenic KK.Cg-\(A^{+/+}\) mouse strains show no significant differences in body weight and adiposity throughout this same period. Measurements of circulating glucose and triglycerides suggest that KK.Cg-\(A^{+/+}\) mice are significantly more diabetic and hypertriglyceridemic than the parental KK strain, but these phenotypes are not observed when the \(A^{+/+}\) mutation is congenic on the B6 genetic background. Thus, we conclude that the effect of the \(A^{+/+}\) mutation is to confer increased adiposity on the B6 genetic background but increased diabetes (with corresponding insulin resistance) and hypertriglyceridemia on the KK genetic background. This phenotypic difference occurs despite the fact that the \(A^{+/+}\) mutation confers hyperphagia to both mouse strains, although again strain differences are apparent. A 20% increase in food intake is observed for B6. Cg-\(A^{+/+}\) mice relative to B6, but a larger 45% increase is observed in KK.Cg-\(A^{+/+}\) mice relative to KK.

Previous reports identify an extract derived from Russian tarragon (PMI-5011) as being an in vivo insulin sensitizer leading to significant reductions in fasting insulin in the treatment of the metabolic syndrome using the KK-\(A^{+}\) mouse model of type 2 diabetes mellitus [17]. Our earlier studies (data not shown) revealed that PMI-5011 did not have a significant effect on glucose, insulin, or adiposity in high-fat–fed obese B6 mice. Thus, to determine if there was a background strain–specific botanical effect, we compared the effectiveness of PMI-5011 treatment in 10-week–old B6. Cg-\(A^{+/+}\) and KK.Cg-\(A^{+/+}\) mutant male mice (Fig. 2). PMI-5011 feeding reduced circulating insulin concentrations in KK.Cg-\(A^{+/+}\), but not in B6.Cg-\(A^{+/+}\) mice. No effects on circulating glucose were noted in either of the 2 mouse models. Thus, this data suggest that the insulin-sensitizing effects of PMI-5011 are mouse background strain specific.

Genetic background effects on phenotypic traits are extraordinarily common in mouse genetic studies. It plays a significant role in determining the overall susceptibility of the individual to tumor susceptibility and regulates disease occurrence and progression, response to pharmaceuticals, and overall health status presumably because of allelic variation in functionally important modifier genes. These genes can influence phenotypes in subtle or profound ways; and the effects provide clues to the underlying pathways, networks, and systems that control biological traits [18,19].

Given the large degree of genetic variation observed in humans, understanding botanical-genotype interactions has

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**Fig. 1. Comparison of the effects of the \(A^{+}\) mutation when congenic on the KK and B6 genetic backgrounds.** Male mice (\(n = 6\)) were purchased from Jackson Laboratory (Bar Harbor, ME) at 4 weeks of age and fed a defined low-fat diet, D12329 (Research Diets), throughout the experiment. Symbols: B6, ▲; B6.Cg-\(A^{+/+}\), ■; KK/HJ (KK), Δ; KK.Cg-\(A^{+/+}\), □. Time zero corresponds to mice having reached 7 weeks of age. Body weight (A) was measured weekly, and body composition (B) was measured monthly. No differences were observed between parental and mutant mouse strains in fat-free mass (not shown). Adiposity is calculated as mass of fat divided by body weight, expressed as a percentage. C, The circulating glucose concentrations measured in plasma recovered from the retroorbital sinus of mice that had been fasted for 4 hours (10:00 AM to 2:00 PM). D, A comparison of triglyceride (white bars), total cholesterol (gray bars), and high-density lipoprotein cholesterol concentrations (striped bars) in the 4 mouse strains, separately measured in 15 \(\mu\)L of whole blood using a Cardiocheck Bioscanner 2000 (Polymer Technology Systems, Inc, Indianapolis, IN) in 4-hour–fasted mice taken after completion of the 12 weeks of phenotypic monitoring.
clear and important health considerations. Inbred mouse strains represent a valuable resource to exploit genetic studies in which allelic variants of important genes can be mapped and ultimately identified.

4. Dietary considerations— the advantages of defined diets over chow

Plant extracts contain a large and complex array of phytochemicals. Thus, the in vivo phenotypic profiles seen in animals fed a diet supplemented with a botanical extract are complicated not only by the possibility of interactions between different phytochemicals present in the same extract but also between one or more phytochemicals and chemical components present in the base diet. In addition, it is well established that dietary macronutrient composition plays a major role in the development of the metabolic syndrome [20-23]. Even for each class of macronutrient, there are differences in the source (animal vs plant) and chemical composition of proteins, carbohydrates (simple vs complex), and fats (unsaturated, monosaturated, cis- and trans-saturated). Undefined chow diets typically contain variable levels of daidzen, genistein, and phytoestrogens [24]. Often, soymeal, alfalfa, and cornmeal are used as dietary supplements; and animal fat is included in some formulations. For these reasons, the BRC uses chemically defined and purified low-fat and high-fat diets for all feeding studies. Standardization of diet has a major advantage in that it promotes a direct comparison of the in vivo effects of different botanicals and purified phytochemicals. The actual defined low-fat diets used by the BRC are D12329 (11% kcal fat; Research Diets, New Brunswick, NJ), used in studies using the spontaneously diabetic and insulin-resistant KK.Cg-A^d/+ mouse, and a matched high-fat and high-sucrose diet, D12331 (58% kcal fat, Research Diets), used for dietary-induced obesity models.

5. Metabolic profiling

Dietary studies involving the use of botanical extracts can take several forms. Feeding young and lean B6 mice with test extracts in the context of a high-fat diet provides information concerning the effectiveness of the extract in regulating weight gain induced by a high-fat diet. However,
first making the mice obese by 3 months of high-fat–diet feeding before initiating feeding with extracts provides useful information on the role of the extract in the treatment of metabolic syndrome phenotypes. It is useful to consider both dietary models to evaluate the same extract in the prevention and treatment of disease. Significant challenges remain in the evaluation of the role of botanical supplements in regulating metabolic syndrome. The concentration of the botanical extract in the diet can be quite variable. Typically, incorporation of the test extract at concentrations greater than 2% to 5% can potentially lead to calorie dilution effects on the base diet, promoting reduced weight gain or increased weight loss depending upon the feeding paradigm being used. We typically measure body composition using nuclear magnetic resonance spectroscopy on live, unanesthetized mice at monthly intervals during these feeding studies (typically 8–12 weeks in duration) using a Bruker mq10 minispec (Bruker Optics Inc, The Woodlands, TX). This approach controls for the possibility of significant changes in body composition occurring in the absence of significant changes in body weight. If nuclear magnetic resonance spectroscopy is not available, then direct measurements of fat pad weights at necropsy can be a suitable surrogate for estimating adiposity.

If changes in body weight or body composition are observed, it is essential that this phenotype be correlated with measurements of food intake conducted in the same mice model before these differences become significantly different. Food intake is measured over a period of 1 week on at least 2 different occasions during the feeding study. It is important to take spillage into account, and this may be significant. Although food intake can be measured in group-housed situations, greater power can be obtained if the animals are singly housed. In this case, feed efficiency (the amount of food eaten for a given increase in body weight) can be measured per mouse. If food intake is unchanged between the treatment and control groups AND effects on body weight or body composition are observed, then these suggest that changes in energy expenditure and/or physical activity of mice in their normal home cages are continuously monitored at 2-minute intervals.

Measurements of the glucose-insulin axis of mice require periodic blood sampling. Glucose concentrations in most murine models can be reliably measured using one of the many types of glucometers available for blood glucose monitoring in diabetic patients. Glucose levels in the whole blood of diabetic KK.Cg-A^+/+ mice, however, can easily exceed 600 mg/dL, beyond the upper calibrated range for many of these instruments. Thus, other methods must be used to measure glucose in plasma or sera. If changes in glucose and/or insulin concentrations are observed, it is often useful to address the functional significance of these changes by performing glucose and insulin tolerance tests. Ultimately, using the services of the National Institutes of Health–supported mouse metabolic phenotyping centers (www.mmpc.org) may be required to perform hyperinsulinemic-euglycemic clamps and thereby obtain useful information on rates of gluconeogenesis and whole-body and tissue-specific glucose disposal.

A goal of the BRC is to identify key signaling pathways in metabolic syndrome that are modified by botanical action. As candidate genes and pathways are identified, it will be necessary to obtain in vivo evidence supporting these observations. The Animal Research Core will select the most appropriate mouse mutants to test in functional assays with the appropriate botanical extract or purified phytochemical. The mouse is an excellent model organism because of the availability of specific mutants that are deficient or otherwise modified in the expression of many genes and proteins. Phenotyping the effects of botanicals in these mutants, many of which are commercially available, allows for the verification of hypotheses generated from other molecular and biochemical approaches.
Acknowledgment/Conflict of Interest

Supported by NIH Grant P50AT002776-01 from the National Center for Complementary and Alternative Medicine (NCCAM) and the Office of Dietary Supplements (ODS), and the National Institutes of Health grants 5P50AT002776-039001 and DK064071 to ARZ. The Russian tarragon plant extracts were provided by Dr David Ribnicky of the Botanical Core of the Botanical Research Center. The author thanks Jacalyn MacGowan and Victoria McRoberts for technical assistance in these studies.

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Botanicals as epigenetic modulators for mechanisms contributing to development of metabolic syndrome

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Abstract

Epigenetics refers to heritable changes in gene expression that are not attributable to changes in DNA sequence and impacts many areas of applied and basic biology including developmental biology, gene therapy, somatic cell nuclear transfer, somatic cell reprogramming, and stem cell biology. Epigenetic changes are known to contribute to aging in addition to multiple disease states. Epigenetic changes can be influenced by environmental factors that in turn can be inherited by daughter cells during cell division and can also be inherited through the germ line. Thus, it is intriguing to consider that epigenetics, in general, may play a role in human conditions that are strongly influenced by changes in the environment and lifestyle. In particular, metabolic syndrome, a condition increasing in prevalence around the world, is one such condition for which epigenetics is postulated to contribute. Epigenetic defects (epimutations) are thought to be more easily reversible (when compared with genetic defects) and, as such, have inspired efforts to identify novel compounds that correct epimutations or prevent progression to the disease state. These efforts have resulted in the development of a rapidly growing new field being referred to as epigenetic therapy. To date, 2 classes of drugs have received the most attention, that is, DNA methyltransferase inhibitors and histone deacetylase inhibitors; but recent data suggest that botanical sources may be a rich source of agents that can potentially modulate the epigenome and related pathways and potentially be useful in attenuating the progression of many factors related to development of metabolic syndrome. This review will provide an overview of the field of epigenetics, epigenetic therapy, and the molecules currently receiving the most interest with respect to treatment, and review data on botanical compounds that show promise in this regard.

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1. Introduction: role of epigenetics in disease

An epigenetic contribution to disease was first realized more than 20 years ago by Feinberg and Vogelstein [1] who distinguished human cancer from normal tissues based on DNA methylation levels. To date, it appears that malignancy represents most of the diseases for which epigenetic defects have been shown to contribute to disease pathogenesis. However, over the recent past, epigenetics has been demonstrated to be a major contributor in the pathogenesis of several other diseases and disorders including schizophrenia and bipolar disorder [2], aging [3], fragile X syndrome [4], leukemia [5], Beckwith-Weideman syndrome [6], and Angelman syndrome [7]. The relevance of epigenetics to more common diseases, for example, diabetes and cardiovascular disease, has been less conspicuous. There is growing consensus however that many conditions that characterize metabolic syndrome, that is, insulin resistance, carbohydrate intolerance, regional adiposity, dyslipidemia, hypertension, and eventual development of cardiovascular disease, are characterized by aberrant “epigenetic programming” during fetal and postnatal development (and possibly as early as preimplantation development). These changes are postulated to be associated with inadequate maternal nutrition and metabolic disturbances during these periods and during their lifetime [8]. Thus, there is great interest to identify
epigenetic-based therapeutic strategies as a means to prevent development and possibly treat these related conditions. Increasing our understanding of epigenetics and the role that molecules such as nutrients and drugs can play in reversing and/or preventing disease-associated epigenetic patterns (eg, de-repressing gene expression) should provide important future directions to develop novel strategies to treat the growing worldwide epidemic of metabolic syndrome.

When one considers molecules that may have an effect on epigenetic patterns, it is interesting to note that plants have been known to harbor regenerative capacity for some time. For example, numerous plants retain plasticity that provides regenerative capabilities [9]. This plasticity, which, for the most part, appears to be generalizable to the plant kingdom, involves epigenetic mechanisms; but limited knowledge is actually available. Both methylation and chromatin changes have been observed. The plant protoplast system appears to be an invaluable experimental tool to further understand mechanisms associated with plasticity and regenerative capability. In this system, terminally differentiated mesophyll cells can be isolated, induced to reenter the cell cycle, and form masses of dividing cells from which roots and stems emerge. The transition of a differentiated mesophyll cell into the cell cycle involves acquisition of pluripotential (dedifferentiation) followed by signal-dependent reentry into the cell cycle, a process also referred to as reprogramming. The plasticity observed in the protoplast system suggests the presence of important molecules and pathways that, if identified and characterized, could be used to induce reprogramming processes as a basis for therapy in humans. The diversity of plants present on this planet also implies a potentially rich source of highly evolved compounds that could be discovered.

2. Mechanisms that regulate epigenetic memory

Epigenetic mechanisms ensure heritable characteristics of cells and functional differences between cell types without changing DNA sequence and is often referred to as cellular memory. Epigenetic mechanisms modify chromatin (DNA and associated proteins) in ways that change the availability of genes to transcription factors required for their expression. Two of the most studied epigenetic phenomena are histone modifications and DNA methylation.

2.1. Histone modifications

The basic building block of chromatin is the nucleosome, which is based on an octamer of histone proteins. Histone tails protrude out of the nucleosome and undergo several posttranslational modifications including acetylation (and deacetylation) and methylation of lysines, phosphorylation, ubiquitylation, sumoylation, adenosine diphosphate ribosylation, glycosylation, biotinylation, and carbonylation [10]. Histone modifications can distinguish euchromatin from heterochromatin and influence associations of proteins and protein complexes that regulate gene transcription or repression by altering the availability of genes to transcription factors. A brief summary of several histone modifications and associated enzymes is presented in Table 1.

2.2. DNA methylation

The DNA molecule can be modified at the 5' position of cytosine rings present in CG (cytosine, guanine) dinucleotide sequences by addition of a methyl group [11]. Patterns of cytosine methylation are distinct for each cell type and confer cell type identity [12]. The DNA methylation patterns are known to be established during mammalian development and subsequently maintained by the maintenance DNA methyltransferases (DNMTs).

The DNA methylation patterns are closely linked to chromatin structure. Unmethylated DNA is typically associated with an active chromatin configuration, whereas methylated DNA is associated with inactive chromatin. The DNMTs catalyze de novo and maintenance DNA methylation. These enzymes catalyze the transfer of a methyl group from the methyl donor S-adenosylmethionine (SAM) onto the 5' position of the cytosine ring found in CpG (CpG notation used to distinguish a cytosine and guanine separated by a phosphate) dinucleotides. Not all CpGs are methylated, and patterns are tissue and time specific. Five enzymes have been identified: DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L.

DNMTs 2 and 3L lack enzymatic function because of an amino-terminal regulatory domain in DNMT2 and the catalytic domain in DNMT3L [13]. DNMT1 is regarded as a maintenance methyltransferase and recognizes hemimethylated DNA. DNMTs 3a and 3b are classified as de novo methyltransferases; they bind to both hemimethylated and unmethylated CpG sites and add methyl groups to previously unmethylated cytosines. DNTM3L has been reported to participate in de novo methylation of retrotransposons [14] and establishing maternal imprints [15].

| Table 1 Classes of histone modifying enzymes |
|-------------|-------------|-------------|-------------|
| Class | Modification | Gene expression | Enzymes |
| HATs | Acetylation | Permissive | Superfamilies: GNAT P300/CBP MYST |
| HDACs | Deacetylation | Repressive | 3 Classes based on localization: I: HDAC1, 2, 3, and 8 II: HDAC4, 5, 6, 7, 9, and 10 III: Surtin enzymes 1-7 |
| HMTs | Methylation | Permissive and repressive | 4 Subgroups: SUV1 SUV2 SUV3 RIZ |
3. Methylation and methyl group metabolism

In the process of methylating molecules such as cytosines in CpG dinucleotides and histones, methyltransferases convert SAM to S-adenosylhomocysteine (SAH) (Fig. 1). Regulating the supply and utilization of methyl groups is accomplished by the enzymatic activity of a key regulatory protein, glycine N-methyltransferase (GNMT). Glycine N-methyltransferase optimizes the ratio of SAM and SAH molecules by catalyzing the conversion of SAM and glycine to SAH and sarcosine [16] (Fig. 1). Because SAH is a potent inhibitor of methyltransferase activity [17], optimizing the SAM/SAH ratio serves to regulate the transmethylation capacity of the cell [18].

4. Methyl group metabolism and relevance to carbohydrate metabolism

Both type 1 and type 2 diabetes mellitus states have been identified as pathological factors in the modulation of methyl group metabolism in rat models [19,20]. Metabolic dysregulation as observed in diabetic states leads to the disruption of hepatic methyl group metabolism, characterized by elevations in GNMT activity and abundance. Up-regulation of GNMT expression and activity would reduce methylation capacity of cells and induce hypomethylation.

5. Epigenetic therapy: correcting epigenetic defects

Epigenetic therapy is a new and rapidly developing area of potential intervention aimed at correcting epigenetic defects. Epigenetic therapy is a potentially very useful form of therapy because epigenetic defects are thought to be more easily reversible when compared with genetic defects. It is also possible that epigenetic therapeutic agents may prevent disease. Currently, several epigenetic-based drugs are undergoing preclinical and clinical trials. Most target various types of cancers such as solid tumors and hematologic malignancies. Targets include enzymes such as histone acetyltransferases (HATs), histone deacetylases (HDACs), DNMTs, and histone methyltransferases (HMTs) [21,22].

6. Epigenetic modifying compounds

Table 2 lists principal classes of epigenetic modifiers and representative compounds within each class. A brief summary follows:

6.1. DNMT inhibitors

There are 2 classes of DNMT inhibitors: nucleoside analogues and non-nucleoside analogues (Table 2). The classic DNMT inhibitor is 5-azacytidine, a derivative of the nucleoside cytidine. The inhibitor was discovered more than 40 years ago [23], and its demethylating activity was discovered subsequently because of its ability to influence cellular differentiation in vitro [24]. 5-Azacytidine is a nucleoside inhibitor that can be incorporated into DNA and can be methylated by DNMTs. The DNMT, however, becomes covalently trapped because the intermediate cannot be resolved, inactivating the enzyme. As the enzyme is depleted, genomic DNA is demethylated as a result of continued DNA replication. To become incorporated into DNA, 5-azacytidine must be modified to a deoxyribonucleoside triphosphate before incorporation into DNA. Before modification, 5-azacytidine can become incorporated into RNA, resulting in a variety of consequences including cytotoxicity. An analogue of 5-azacytidine, 5-aza-2′deoxycytidine (AKA, decitabine), does not require modification and is thought to be less cytotoxic, but still has severe cytotoxic affects [25]. For example, decitabine has been used in clinical trials and has shown promise for treatment of myeloid malignancies [26,27], but also has toxic effects including myelosuppression and neutropenic fever [25].

Several non-nucleoside compounds also inhibit DNMT activity. Two compounds have been reasonably characterized, whereas compounds representing 3 subclasses are less understood. The 2 characterized compounds include (−)-epigallocatechin-3-gallate (EGCG), the key polyphenol in green tea [28], and RG108, a molecule identified in an in silico screening assay [29]. (−)-Epigallocatechin-3-gallate inhibits DNMT activity in protein extracts and human cancer cell lines [30] and is thought to block the active site of DNMT1. Degradation of EGCG results in production of hydrogen peroxide, a strong oxidizing agent [31] that may result in cytotoxicity. In contrast, RG108 appears to have low toxicity and has been demonstrated to inhibit the catalytic activity of recombinant DNMTs. The inhibitory activity appears to be direct and specific for DNMTs [29].

6.2. HDAC inhibitors

The original discovery that trichostatin A (TSA) had antileukemia properties due to inhibition of HDAC enzymes [32] has inspired the discovery of additional HDAC inhibitors that have been proposed as possible therapeutic agents for a variety of diseases. There are 4 classes of HDAC inhibitors: short-chain fatty acids,
hydroxamic acids, cyclic tetrapeptides, and benzamides. Short-chain fatty acids are typically not potent inhibitors of HDACs. In contrast, hydroxamic acids and cyclic tetrapeptides are potent inhibitors of HDACs (for review, see Yoo and Jones [22]).

6.3. HAT inhibitors

Several transcription factors contain HAT activity, and the proteins are grouped into distinct classes based on sequence homologies and functionality [33]. It has been shown that some HAT genes are misregulated in cancer. For example, it has been demonstrated that p300 is an E1A binding protein; and p300 missense and deletion mutations have been identified in breast, colorectal, gastric, and epithelial cancers [34], suggesting that identification of HAT inhibitors may have therapeutic value. To date, relatively few HAT inhibitors have been identified and include the bisubstrate analogues Lys-CoA and H3-CoA-20 [35] and natural small molecules such as arachidonic acid [36], garcinol [37], and curcumin [38]. Synthetic small molecules having HAT inhibitory activity include γ-butyrolactone MB-3 [39], isothiazolones [40], and quinolines MC1823, MC1626, and MC1752 [41-43], of which MC1823 is 10-fold more potent than MC1626 and MC1752.

6.4. HMT inhibitors

To date, identification of inhibitors of HMTs is in its infancy; but it is likely that a growing interest will develop over the next several years.

7. Combining epigenetic therapeutic strategies

Deoxyribonucleic acid methylation and histone modification are tightly linked; and more recently, combined approaches aimed at inhibiting DNMTs as well as HDACs are showing promise as a strategy for cancer treatment. For example, it has recently been reported that the HDAC inhibitor TSA in combination with a low dose of the DNMT inhibitor decitabine induced expression of 4 hypermethylated genes in cultured colorectal carcinoma cells [44]. In addition, a combination of decitabine and the HDAC inhibitor phenylbutyrate had a synergistic effect in preventing lung cancer induced in mice by tobacco carcinogen [45].

8. Botanical extracts as epigenetic modulators

Recently, our laboratory has reported that the peptide nucleoplasmin induces up-regulation of several hundred genes in a somatic cell nuclear transfer reprogramming model [46]. Many of the genes were previously silenced in the somatic cell donor cell line. We also reported that exposure of mammalian somatic cells growing in culture to all-trans retinoic acid up-regulates expression of Gnmnt, inducing global demethylation, increased expression of the pluripotency gene Oct4, and restoration of differentiation potential [47]. Moreover, we have reported that down-regulating Dnmt1 and/or 3b of cells in culture has similar effects [47]. These results indicate that compounds or activities can be identified that target key components of epigenetic pathways and alter gene expression patterns typically thought to be “fixed” or permanent. As stated previously, it has been recognized that plants are characterized by remarkable plasticity that is associated with epigenetic changes. Thus, we have been investigating whether activities could be identified in plants that could also target expression of key epigenetic regulatory genes in mammalian cells. Our initial screens have focused on Gnmnt,
Dnmt1, and Dnmt3b gene expression because changes in expression of these genes have been demonstrated by us and others to induce alterations in DNA methylation and gene expression. In addition, we have assessed expression of the pluripotency gene Nanog, typically silenced or expressed at very low levels in mammalian somatic cells and associated with promoter hypermethylation. To date, we have screened approximately 50 botanical extracts; and extracts were evaluated using NIH/3T3 fibroblast cells cultured in 6-well culture dishes. The particular botanicals evaluated consisted of extracts of *Allium tuberosum* L, for example, Chinese chives or garlic chives; *Ligustrum lucidum* L, for example, glossy privet; and *Artemisia dracunculus* L, for example, Russian tarragon. Extracts of *A. tuberosum* L, that is, ALT-S, and *L. lucidum* L, NZ-01 (assessed at concentrations ranging from 100 μg/mL to 1 mg/mL for 48 and 96 hours and 1 and 2 weeks), were obtained from the Louisiana State University Agricultural Center (Baton Rouge, LA), whereas subfractions of *A. dracunculus* L, that is, PMI 5011 (evaluated at concentrations of 10 and 100 μg/mL for 48 and 96 hours), were obtained from the Botanical Core Laboratory of the Botanical Research Center of the Pennington Biomedical Research Center and Rutgers University. The cells were harvested at the conclusion of the time points, and messenger RNA was obtained using the RNeasy Mini Kit (Qiagen, Valencia, CA). Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) was performed to examine the relative expression levels of the genes Gmmt, Dnmt1, Dnmt3b, and Nanog as compared with Gapdh. Results from the 3 botanical sources (PMI 5011, NZ-01, and ALT-S) are described.

PMI 5011 (*A. dracunculus* L) was originally identified from a screening of extracts for hypoglycemic activity in diabetic mice as the most promising candidate for the development of a nutritional supplement for diabetes and is described in detail in other articles as part of this supplement. The active compounds in the preparation are believed to be members of the sesquiterpene lactone or flavanoid groups, of which the *Artemisia* family is well known. Preliminary data further suggest that PMI 5011 may have significant effects to improve carbohydrate metabolism by enhancing molecular events of insulin action in skeletal muscle. We have identified 3 subfractions that down-regulate Dnmt1 or Dnmt3b and one subfraction that up-regulates Nanog expression (Fig. 2A–D). Specifically, exposure of NIH3T3 cells to 100 μg/mL of subfraction 7-6-2 for 48 hours reduced
DNMT1 gene expression (P < .05) (Fig. 2A). Similar decreases were observed after exposure to 100 μg/mL of subfraction 7-6-5 for 48 and 96 hours (P < .05) (Fig. 2B).

Exposure of NIH3T3 cells to 1 μg/mL of subfraction 222 reduced Dnmt3b expression (P < .05) (Fig. 2C). In contrast, Nanog expression was up-regulated (P < .05) in NIH3T3 cells after exposure to 1 and 10 μg/mL of PMI 5011 subfraction 222-1 for 96 hours (Fig. 2D). No changes in Gmmt expression were detected after treatment with any of the subfractions.

ALT-S (A tuberosum L) was identified in a random screen from a collection of a variety of botanicals located at the Louisiana State University. Since that time, we have learned that A. tuberosum is being investigated for anticancer activity [48]. We treated NIH3T3 cells with 100-μg/mL or 1-mg/mL extracts of ALT-S for 48 or 96 hours. Quantitative RT-PCR demonstrated down-regulation of both Dnmt1 and Dnmt3b (P < .05) at 48 hours after treatment with 100 μg/mL (Fig. 3A, B).

NZ-01 (L. lucidum L) was also identified in our random screen of botanicals and has also been reported to have antidiabetic [49], anticancer [50], antioxidant [51], and neuroprotective [52] activities. Up-regulation of Gmmt gene expression was detected in NIH3T3 cells by quantitative RT-PCR after treatment with 750 μg/mL of NZ-01 extract for 48 and 96 hours (P < .05) as well as 1 mg/mL for 96 hours.

Fig. 3. A, Effects of botanical extract ALT-S-C on relative expression levels of Dnmt1 as compared with Gapdh. Significantly decreased expression in NIH/3T3 murine fibroblast cells that were treated with crude botanical extract ALT-S-C at 100 μg/mL and 1 mg/mL for 48 hours. Fold decrease shown in relation to untreated, control cultures. Statistical analyses were performed by a 2-sample, equal-variance, 2-tailed t test. *P < .05. B, Effects of botanical extract ALT-S-C on relative expression levels of Dnmt3b as compared with Gapdh. Significantly decreased expression in NIH/3T3 murine fibroblast cells that were treated with crude botanical extract ALT-S-C at 100 μg/mL and 1 mg/mL for 48 hours. Fold decrease shown in relation to untreated, control cultures. Statistical analyses were performed by a 2-sample, equal-variance, 2-tailed t test. *P < .05.

Fig. 4. A, Effects of botanical extract NZ-01 on relative expression levels of Gmmt as compared with Gapdh. Expression in NIH/3T3 murine fibroblast cells that were treated with 100 μg/mL, 250 μg/mL, 500 μg/mL, 750 μg/mL, and 1 mg/mL of crude extract NZ-01 for 48 hours, 96 hours, 1 week, and 2 weeks. Significant increase shown in relation to untreated, control cultures. Statistical analyses were performed by a 2-sample, equal-variance, 2-tailed t test. *P < .05; **P < .01. B, Effects of botanical extract NZ-01 on relative expression levels of Dnmt1 as compared with Gapdh. Expression in NIH/3T3 murine fibroblast cells that were treated with 100 μg/mL, 250 μg/mL, 500 μg/mL, 750 μg/mL, and 1 mg/mL of crude extract of NZ-01 for 48 hours, 96 hours, 1 week, and 2 weeks. Significant increases and decreases shown in relation to untreated, control cultures. Statistical analyses were performed by a 2-sample, equal-variance, 2-tailed t test. *P < .05; **P < .01; ***P < .001. C, Effects of botanical extract NZ-01 on relative expression levels of Nanog as compared with Gapdh. Expression in NIH/3T3 murine fibroblast cells that were treated with 100 μg/mL, 250 μg/mL, 500 μg/mL, 750 μg/mL, and 1 mg/mL of crude extract of NZ-01 for 96 hours. Significant increase shown in relation to untreated, control cultures. Statistical analyses were performed by a 2-sample, equal-variance, 2-tailed t test. **P < .01; ***P < .001.
Acknowledgment/Conflict of Interest

This work was supported in part by NIH Grant P50AT002776-01 from the National Center for Complementary and Alternative Medicine (NCCAM) and the Office of Dietary Supplements (ODS).

References


Effects of soy protein and isoflavones on insulin resistance and adiponectin in male monkeys

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Abstract

Isoflavones may influence insulin action by means of their well-known receptor-mediated estrogenic activity. However, isoflavones also bind to peroxisome proliferator–activated receptors (PPARs) that are strongly associated with insulin action. Soy protein with its isoflavones has previously been shown to improve glycemic control in diabetic postmenopausal women and to improve insulin sensitivity in ovariectomized monkeys. The purpose of the current report was to extend our studies of dietary soy protein to male monkeys and determine effects of the soy isoflavones on insulin resistance. Two studies are reported here. Study one involved 91 male monkeys consuming 3 diets differing only by the source of protein (casein-lactalbumin, soy protein with a low isoflavone concentration, or soy protein with a high isoflavone concentration). Intravenous glucose tolerance tests were done, and plasma adiponectin and lipoprotein concentrations were determined after 25 months of study. Samples of visceral fat were obtained at 31 months for assessment of adiponectin and PPARγ expression. The second study involved 8 monkeys in a Latin-square design that compared the effects of diets with casein/lactalbumin, soy protein with a high isoflavone concentration, or soy protein that was alcohol-washed to deplete the isoflavones. After 8 weeks of treatment, insulin sensitivity and plasma lipoproteins were assessed. At 10 weeks, a biopsy of the skeletal muscle was performed for determination of insulin receptor, PPARα, and PPARγ content. The major findings were that consumption of isoflavone-containing soy protein dose-dependently increased insulin responses to the glucose challenge and decreased plasma adiponectin, whereas isoflavone-depleted soy protein decreased body weight and had no effect on plasma adiponectin concentrations. Muscle PPARα and γ expression was also increased with the isoflavone-depleted soy relative to either casein or soy protein containing the isoflavones. Further studies are needed to determine the mechanisms involved in these effects of a high–soy isoflavone diet and to optimize dietary isoflavone content for maximal health benefits in male subjects.

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1. Introduction

Current estimates are that 50% of the adult population of the United States is obese [1]. About half of the obese population also has prediabetes or the metabolic syndrome [2]. Insulin resistance and obesity are key features of the metabolic syndrome and type 2 diabetes mellitus (T2DM) [2,3]. One potential mechanism involves the production of hormones or adipokines by adipose tissue. Plasma concentrations of many adipokines, such as leptin, tumor necrosis factor–α, and plasminogen activator inhibitor–1, have been associated positively with insulin resistance, whereas adiponectin is associated negatively [4,5]. Furthermore, lower plasma concentrations of adiponectin are associated with increased incidence of metabolic syndrome, diabetes, and vascular disease [5-7].

Pharmacologic agents, such as thiazolidinediones, are potent agonists of peroxisome proliferator–activated receptor (PPAR) γ and have become useful clinical tools to improve insulin resistance and raise adiponectin concentrations [5,7]. Interestingly, the isoflavones genistein and daidzein, plant estrogens found in soy beans and processed soy protein, have also been shown to bind to PPARγ as well as PPARα and δ [8-10], suggesting the potential value of
isoflavones as a nutritional approach to modulating insulin action. Soy is the most commonly used botanical in the United States; and the Food and Drug Administration has approved a health claim for soy protein and soy-based food products based largely on the evidence that soy consumption improves plasma lipid and lipoprotein concentrations and might reduce risk of coronary heart disease, yet does not appear to increase cancer risk [11].

The earliest report of soy beans having beneficial effects on glycemic control was in 1910 [12], when the consumption of soy beans was found to decrease glycosuria in diabetics. Until relatively recently, however, there have been few studies of the effects of soy or its isoflavones on glycemic indices. Among the somewhat limited data is the finding that consumption of soy protein containing isoflavones was associated with improved lipoprotein and glycemic control in T2DM postmenopausal women [13]. In nondiabetic postmenopausal women, soy consumption was associated with decreased fasting insulin concentrations; and isoflavone intake was inversely associated with postchallenge insulin concentrations [14]. Similarly, we have found improved plasma lipoprotein profiles and insulin sensitivity in premenopausal monkeys fed soy-rich diets [15]. However, in smaller studies of predominantly male T2DM subjects (14 men and 6 women), the improvement in lipoprotein profiles has been seen, but not the improved glycemic control [16]. Similarly, in a small study of diabetic and nondiabetic male monkeys, soy protein improved plasma lipoproteins and atherosclerosis but did not affect glycemic control [17].

These few studies suggest that the benefits of soy on carbohydrate metabolism are more apparent in female than male subjects. The studies did not assess potential mechanisms involved with changes in insulin action or whether the effect is due to the soy protein, its isoflavones, or both. In addition to the isoflavones binding to PPAR, they have estrogenic activity, binding to both estrogen receptor (ER) α and ERβ, but with greater affinity to ERβ [11]. Genistein is also a tyrosine kinase inhibitor [18], so high concentrations may inhibit insulin signaling pathways.

The purpose of the current studies was to extend our studies of dietary soy protein and isoflavones to address their effects on insulin resistance in male subjects. Furthermore, because adiponectin is strongly associated with insulin action and is also regulated by PPARγ2, we explored whether changes in insulin resistance were related to changes in plasma concentrations of this adipokine.

2. Methods

2.1. Animal studies

2.1.1. Study 1

Ninety-one adult male cynomolgus monkeys (Macaca fascicularis) were imported from Indonesia (Institut Pertanian Bogor). Effects on plasma lipoprotein and isoflavone concentrations and the cardiovascular system have been reported previously [19]. All monkeys consumed a Western-type diet differing only by the source of dietary protein [19]. The major source of protein was casein-lactalbumin (casein, n = 30) for group 1, a mixture of unmodified soy protein isolate and alcohol-washed (isoflavone-depleted) soy protein isolate approximating human intake of 75 mg isoflavones per day (low ISO, n = 30) for group 2, and unmodified soy protein isolate containing an amount approximating human intake of 150 mg isoflavones per day (high ISO, n = 31) for group 3. Other than protein source, diets were equal in macronutrients, with 19% of calories from protein, 35% from lipid (0.28 mg cholesterol per kilocalorie), and 46% from carbohydrates. Detailed descriptions of the diet compositions have been published previously [19]. Glucose and insulin responses to an intravenous glucose tolerance test (IVGTT) and plasma adiponectin concentrations were determined after 25 months of study as described previously [20]. Visceral fat samples were collected after 31 months of treatment.

2.1.2. Study 2

Eight old, obese, hyperinsulinemic male monkeys were used in this study (note increased body weight and fasting insulin of monkeys in Table 2 vs Table 1). The study was a 3-phase Latin-square design, such that each monkey received each diet for a baseline period during which animals consumed the control diet. Animals were randomized to one of 3 diet groups containing (1) casein, (2) alcohol-washed (isoflavone-depleted) soy (SOY−, 8 mg isoflavones per day human equivalent), or (3) intact soy protein (SOY+, 132 mg isoflavones per day human equivalent). Diets were equal in macronutrients with the exception of the protein source and were composed of 19% of calories from protein, 20% from lipid (0.19 mg cholesterol per kilocalorie), and 61% from carbohydrates. Each phase lasted 10 weeks.

The first phase of diet interventions began the week after baseline measures were completed. After 4 and 8 weeks of treatment, measurement of body weight and blood collection for analysis of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) concentrations were done. At 8 weeks, minimal model analysis (frequently sampled IVGTT) was performed [21]. At 10 weeks of treatment, animals were sedated for measurement of body weight, plasma lipids, and lipoproteins, and for muscle biopsy of the vastus lateralis muscle.

All procedures involving animals were conducted in compliance with state and federal laws, standards of the US Department of Health and Human Services, and guidelines established by the Institutional Animal Care and Use Committee.

2.2. Clinical chemistry measures

Animals were fasted overnight and sedated with ketamine hydrochloride (15 mg/kg intramuscularly) (Ketaset; Fort...
Dodge Animal Health, Fort Dodge, IA) before blood collection. Total cholesterol, HDL-C, and TG were determined by enzymatic techniques [19]. Lipoprotein fractions were separated by ultracentrifugation and high-performance liquid chromatography, and the cholesterol content of each fraction was determined enzymatically [19]. Plasma glucose, fructosamine, insulin, C-peptide, leptin, and adiponectin were determined as described [20–22].

Measurements for the IVGTT included the glucose area under the curve (AUC), calculated as the total area including all time points; the disappearance rates (K value) for glucose and insulin were calculated from the linear portion of the curve [20]. Measurements for the frequently sampled IVGTT included the insulin sensitivity index as described previously [15,21].

### 2.3. Western blot analyses

Biopsies of visceral fat (study 1) and skeletal muscle (study 2) were snap frozen in liquid nitrogen and stored at –70°C until processed as described previously [23]. Fat samples were assessed for adiponectin (mouse monoclonal antiadiponectin; BioVision Research Products, Mountain View, CA) and PPARγ (rabbit polyclonal anti-PPARγ; Santa Cruz Biotechnology, Santa Cruz, CA). Skeletal muscle homogenates were assessed for insulin receptor (IR) expression (mouse monoclonal anti-IR; Research Diagnostics, Flanders, NJ). Basal IR activity was determined using a phosphorylation state–specific antibody generated against the phosphorylated tyrosine residue 1158 of the human IR (anti-IRpY1158; Biosource International, Camarillo, CA). The blots were also probed with anti-PPARα (rabbit polyclonal anti-PPARα, Santa Cruz Biotechnology) or anti-PPARγ antibody (rabbit polyclonal anti-PPARγ; Biomat International, Plymouth Meeting, PA). To account for equal protein loading, blots were stripped (Re-Blot Plus; Chemicon International, Temecula, CA) and reprobed for actin in muscle (monoclonal actin Ab-1; Oncogene Research Products, Boston, MA) or guanidine disassociation inhibitor in fat (rabbit anti-Rho guanidine disassociation inhibitor polyclonal antibody, Santa Cruz Biotechnology) [24]. As described previously [23], signals were detected using a Storm Phosphorimager 860 (Molecular Dynamics, Sunnyvale, CA); and densitometry was quantified using ImageQuant Software (Version 5.2; Amersham Biosciences; Sunnyvale, CA). Densitometry results are presented as arbitrary scanning units after correcting for loading; however, results were similar regardless of this correction.

### 3. Statistics

All data are reported as mean ± SEM.

#### 3.1. Study 1

The glucose tolerance test outcomes (glucose response curve and insulin response curve) were analyzed using linear mixed effects models. Other outcomes were analyzed using 1-way analysis of variance, adjusting for baseline measure when available.

#### 3.2. Study 2

As the study design was a Latin-square design, treatment means were assessed for time trends, treatment by phase interactions, and homogeneity of variance (Levene). Data were analyzed for the difference between treatments by paired t test because there were no treatment by period interactions. Analyses were performed by SAS 9.1 (Cary, NC).

### 4. Results

#### 4.1. Study 1

There were no treatment effects on fasting glucose, insulin, or overall glycemic control as assessed by fructosamine concentrations (Table 1). As reported previously [19], plasma low-density lipoprotein cholesterol (LDL-C) was decreased by 21% and 17% and HDL-C was increased by 36% and 18% in the groups fed low ISO and high ISO, respectively (all Ps < .05). Plasma TG was unaffected [19]. Fig. 1 depicts the glucose and insulin responses to the IVGTT. There was no treatment effect on glucose responses (Fig. 1 and Table 1). However, insulin responses were significantly increased (P < .05) with treatment (Fig. 1), with a dose-dependent increase in the maximal insulin response (P = .01).

The ratio of insulin-glucose AUC, an index of insulin resistance, was 17% and 41% greater with increasing dietary isoflavone content (); but this did not reach statistical significance (P > .05). Male monkeys fed high ISO also tended to gain more weight (Table 1). Consistent with an insulin-resistant condition, plasma adiponectin concentra-

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### Table 1

Study 1: measures (mean ± SEM) for male monkeys consuming protein from casein-lactalbumin (casein), soy with low isoflavones (Low Iso Soy), or soy with high isoflavones (High Iso Soy)

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Casein (n = 30)</th>
<th>Low Iso Soy (n = 30)</th>
<th>High Iso Soy (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL), fasting</td>
<td>74.5 ± 1.68</td>
<td>73.6 ± 1.94</td>
<td>74.2 ± 1.91</td>
</tr>
<tr>
<td>Glucose, IVGTT, max</td>
<td>398.4 ± 6.59</td>
<td>399.5 ± 7.82</td>
<td>416.1 ± 6.80</td>
</tr>
<tr>
<td>Glucose, IVGTT, K value</td>
<td>3.63 ± 0.19</td>
<td>3.55 ± 0.21</td>
<td>3.87 ± 0.23</td>
</tr>
<tr>
<td>Insulin (IU/mL), fasting</td>
<td>28.00 ± 3.73</td>
<td>27.30 ± 3.55</td>
<td>28.92 ± 9.00</td>
</tr>
<tr>
<td>Insulin, IVGTT, max</td>
<td>115.6 ± 10.36</td>
<td>134.7 ± 12.49</td>
<td>172.4 ± 14.96*</td>
</tr>
<tr>
<td>Fructosamine, baseline</td>
<td>263.9 ± 6.12</td>
<td>272.9 ± 6.28</td>
<td>271.2 ± 6.05</td>
</tr>
<tr>
<td>Fructosamine, treatment</td>
<td>260.1 ± 6.53</td>
<td>252.7 ± 4.61</td>
<td>253.1 ± 6.59</td>
</tr>
<tr>
<td>Body weight (kg), baseline</td>
<td>5.51 ± 0.12</td>
<td>5.70 ± 0.13</td>
<td>5.68 ± 0.13</td>
</tr>
<tr>
<td>Body weight (kg), treatment</td>
<td>5.95 ± 0.14</td>
<td>6.05 ± 0.16</td>
<td>6.22 ± 0.17</td>
</tr>
<tr>
<td>Fat adiponectin (ASU)</td>
<td>0.58 ± 0.07</td>
<td>0.58 ± 0.08</td>
<td>0.58 ± 0.08</td>
</tr>
<tr>
<td>Fat PPARγ (ASU)</td>
<td>10.80 ± 0.52</td>
<td>9.70 ± 0.78</td>
<td>11.20 ± 1.05</td>
</tr>
</tbody>
</table>

ASU indicates arbitrary standardized unit.

* P < .05 vs casein.
tions (Fig. 2) were significantly decreased with isoflavone consumption ($P = .02$).

Despite the lower plasma adiponectin concentrations with soy isoflavones, there was no difference in the abundance of adiponectin in fat, the primary source of circulating adiponectin. Adiponectin levels are controlled in part by PPAR$\gamma$, the expression of which was also not affected by soy isoflavones (Table 1).

4.2. Study 2

Treatment with SOY$-$ decreased body weight compared with SOY$+$ ($P = .02$) but not casein ($P = .17$) (Table 2, Fig. 3), despite no effect on leptin concentrations (Table 2). Compared with casein, SOY$-$ treatment resulted in lower LDL-C ($P = .02$) but had no effect on TG or HDL-C. There was no significant effect of SOY$+$ treatment on LDL-C, HDL-C, or TG compared with casein. There was also no significant effect of SOY$-$ or SOY$+$ treatment on insulin sensitivity, glucose effectiveness, fasting blood glucose, insulin, or C-peptide concentrations compared with casein (all $P$s $>.05$).

SOY$-$ treatment increased PPAR$\alpha$ and PPAR$\gamma$ expression in skeletal muscle compared with casein ($P = .009, P = .03$); but SOY$+$ treatment had no effect ($P = .61, P = .86$).

![Fig. 1. Glucose (top) and insulin (bottom) responses to an IVGTT for monkeys consuming casein, soy protein with low isoflavone dose (Low Iso Soy), and soy protein with high isoflavone dose (High Iso Soy). There were no changes in glucose response, but a dose-dependent increase in insulin responses was found with isoflavone intake (analysis of variance, $P < .05$). The treatment differences were due to High Iso Soy compared with casein ($P = .03$) and an intermediate response with Low Iso Soy compared with casein ($P = .11$).](image1)

![Fig. 2. Changes in insulin resistance (as determined by insulin-glucose AUCs) after a glucose challenge (as depicted in Fig. 1) and plasma adiponectin concentrations with monkeys consuming casein, Low Iso Soy, and High Iso Soy. Adiponectin was significantly less in Low Iso compared with casein ($P = .02$), with a similar trend for High Iso compared with casein ($P = .08$).](image2)
suggesting that isoflavones attenuated the effect of soy protein. There were no significant correlations between PPARα and PPARγ expression and insulin sensitivity, or fasting glucose and insulin concentrations, or body weight.

SOY+ treatment resulted in less skeletal muscle IR expression compared with casein treatment (\(P = .01\)), with a similar tendency for SOY− treatment (\(P < .05\)). However, there was no difference in basal IR activity (tyrosine phosphorylation) with either of the soy treatments (Table 2).

5. Discussion

The major findings from these studies are that, in male monkeys, consumption of soy protein with its isoflavones increases insulin secretion after a glucose challenge (Fig. 1). Despite the increased insulin secretion, there were no changes in glucose disposal and a dose-dependent increase in the ratio of insulin-glucose AUCs determined from the IVGTTs (Fig. 2), indicating an increase in peripheral insulin resistance due to the isoflavones. Furthermore, consistent with an insulin resistance state, there is a significant decrease in plasma adiponectin concentrations with soy isoflavones (Fig. 2). In contrast, consumption of isoflavone-depleted soy protein resulted in loss of body weight and no effect on plasma adiponectin concentrations. Muscle PPARα and γ expression was also increased in this group compared with either casein or soy protein containing the isoflavones.

The increase in insulin secretion after the glucose challenge was not unexpected. As earlier studies have shown with estradiol [25], studies of genistein [26,27] and, to a lesser extent, daidzein [27] found increased insulin secretion from islet preparations. A more recent study by Liu et al [28] found that genistein increases glucose-stimulated insulin secretion in cell lines and mouse pancreatic islets at micromolar concentrations via a cyclic adenosine monophosphate–dependent protein kinase mechanism. This action could be beneficial and may be the basis for the early report suggesting clinically relevant decreases in glucosuria in diabetics [12], consistent with a secretagogue-like effect. However, in the current studies, the lack of increased glucose removal despite the increased insulin secretion indicates peripheral insulin resistance.

Potential mechanisms for increased insulin resistance could relate to the fact that genistein is a potent tyrosine kinase inhibitor for both platelet-derived growth factor and epidermal growth factor [18,29]. Our data (Table 2) and those of others [30] suggest that basal IR activity is not affected by soy isoflavones. However, high concentrations of genistein could be inhibitory, whereas daidzein, which is not a tyrosine kinase inhibitor, likely would not be inhibitory.

Other postulated mechanisms that could increase peripheral insulin resistance include changes in insulin receptor number, affinity, intracellular phosphorylation, and altera-

Table 2

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Casein (n = 8)</th>
<th>SOY− (n = 8)</th>
<th>SOY+ (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL), fasting</td>
<td>67.8 ± 4.8</td>
<td>81.9 ± 6.0</td>
<td>69.9 ± 5.2</td>
</tr>
<tr>
<td>Insulin (IU/mL), fasting</td>
<td>54.3 ± 8.6</td>
<td>53.0 ± 16.5</td>
<td>42.5 ± 8.8</td>
</tr>
<tr>
<td>C-peptide (ng/mL)</td>
<td>9.87 ± 2.7</td>
<td>7.31 ± 3.1</td>
<td>6.83 ± 1.7</td>
</tr>
<tr>
<td>SI (10−3 min−1 mL−1)</td>
<td>3.10 ± 1.15</td>
<td>3.19 ± 0.85</td>
<td>2.85 ± 1.11</td>
</tr>
<tr>
<td>Glucose effectiveness (min−1)</td>
<td>0.037 ± 0.010</td>
<td>0.036 ± 0.014</td>
<td>0.020 ± 0.006</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>71.8 ± 11.6</td>
<td>71.3 ± 12.1</td>
<td>71.5 ± 16.5</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>106.1 ± 18.5</td>
<td>78.9 ± 15.4*</td>
<td>96.3 ± 16.1</td>
</tr>
<tr>
<td>Adiponectin (ng/mL)</td>
<td>4.26 ± 1.39</td>
<td>5.42 ± 2.08</td>
<td>3.96 ± 1.43</td>
</tr>
<tr>
<td>Fasting leptin (ng/mL)</td>
<td>53.5 ± 11.9</td>
<td>40.6 ± 5.2</td>
<td>40.3 ± 6.4</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>9.99 ± 0.43</td>
<td>9.53 ± 0.55**</td>
<td>10.22 ± 0.47</td>
</tr>
<tr>
<td>Muscle IR activity (ASU)</td>
<td>16794 ± 645</td>
<td>31387 ± 3350</td>
<td>29454 ± 5317</td>
</tr>
<tr>
<td>Muscle IR expression (ASU)</td>
<td>4726 ± 820</td>
<td>2879 ± 268</td>
<td>2721 ± 596*</td>
</tr>
</tbody>
</table>

SI indicates insulin sensitivity index.
* \(P < .05\) vs casein.
** \(P < .05\) vs SOY+.
tions in the glucose transport apparatus [31-36]. Insulin receptor number was found to be decreased in rat livers perfused with genistein [31]. This result is consistent with the in vivo finding reported here for skeletal muscle (Table 2). Other effects that could be detrimental to insulin action include genistein-induced inhibition of Glut4 translocation in rat adipocytes [32] and effects on glucose oxidation [30]. These inhibitory effects on hormone signal transduction could be due to inhibition of other protein kinases, such as those with adenosine triphosphate binding at the catalytic sites [30]. In support of this, genistein has been shown to inhibit Akt kinase activity [33]; and effects of soy diet on Akt activity has also been shown to result in worsening of heart disease in male but not female mice [34].

In vitro studies have shown that soy isoflavones increase expression of PPARs [8-10]. In murine macrophage-like RAW 264.7 cells expressing a peroxisome proliferator response element–containing reporter and either PPARα or PPARγ plasmids, unconjugated genistein and daidzein increased both PPARα- and γ-directed gene expression [10]. Consistent with a PPARγ effect, when obese Zucker rats were fed diets containing soy protein with isoflavones, the animals had improved lipid metabolism and glucose tolerance; but they gained weight consistent with PPARγ agonist treatment [10].

Genistein (>1 μm) was also shown to act as a ligand for PPARγ in mesenchymal progenitor cells (precursor cells for osteoblasts and adipocytes), resulting in up-regulation of adipogenesis and down-regulation of osteogenesis. Transfection experiments showed that activation of PPARγ by genistein at micromolar concentrations down-regulates its estrogenic transcriptional activity, whereas activation of ERα and ERβ by genistein down-regulates PPARγ transcriptional activity [8]. These same investigators reported similar effects with daidzein [9]. In addition, there were concentration-dependent biphasic effects of daidzein on osteogenesis and adipogenesis that were not apparent when ERs were blocked. As well as transactivating PPARγ, daidzein also transactivated PPARα and δ. These studies suggest cross talk between ER and PPAR, with outcomes dependent on the balance between activated ERs and PPARγ.

The PPAR action is modified by cofactors such as PPARγ coactivator–1 (PGC-1). The PGC-1 is also estrogen responsive and may mediate some of the ER transcriptional effects [37]. As isoflavones also have estrogenic activity, some effect on glycemic control may be mediated through PGC-1 [38]. Taken together, these studies suggest an intriguing mechanism pathway whereby soy isoflavones and endogenous hormones may interact to affect PPAR action, resulting in different action in male and female subjects.

Because genistein and daidzein have both been shown to bind to and activate PPARγ [8-10], it is likely that changes in insulin sensitivity could then be modified by adiponectin, which is increased in response to PPARγ agonists [5-7]. Studies in mice have shown that soy protein isolate containing isoflavones increased both plasma concentrations and adipose tissue messenger RNA abundance of adiponectin [39,40]. This is opposite of our finding in monkeys of lower plasma adiponectin concentrations with no difference in adipose tissue expression (Fig. 2, Table 1). The only data we are aware of in humans suggest that soy isoflavones do not affect plasma adiponectin concentrations [41]. Interestingly, low adiponectin levels have been shown to be associated with impaired vasodilation in people [6]. In this study, soy isoflavones did not improve arterial vasodilation in male monkeys [19]; but plasma lipids and atherosclerosis were improved with soy isoflavones in this study [19] and those of others [11].

There are also sex-specific differences in metabolism of soy isoflavones. Stroud et al [42] found that male monkeys had higher plasma genistein, daidzein, and total isoflavones concentrations compared with premenopausal female monkeys fed the same soy isoflavone-containing diet. It is not known how these varying plasma isoflavone concentrations relate to different tissue levels, but it is likely that tissue differences occur.

Although soy isoflavones have often been thought to be the active, beneficial ingredient of soy beans, others have proposed components of soy protein to be the health-beneficial component. For example, Moriyama et al [43] suggest that soy protein, in particular the 7S component, has therapeutic benefits for treatment of obesity and metabolic syndrome. Likewise, Lovati et al [44] have shown beneficial effects with the 7S component on lipoprotein metabolism. Thus, it is likely that, although soy protein both is heart-healthy and improves a number of aspects of the metabolic syndrome, these effects are attenuated by the soy isoflavones in male but not necessarily in female subjects. We show here that the isoflavones resulted in a dose-dependent increase in insulin resistance in male monkeys (Fig. 1) and lower plasma adiponectin concentrations (Fig. 2). However, the smaller study with isoflavone-depleted soy (study 2) resulted in lower body weight (Fig. 3) and greater muscle PPARα and γ expression (Fig. 4). Future studies with a comparison of soy

![Fig. 4. The PPARγ and PPARα expression in skeletal muscle of monkeys with consumption of casein, SOY−, and SOY+ after a 10-week period using a Latin-square design. Expression is significantly greater (P < .05) for SOY− compared with casein.](image-url)
protein, in particular the 7S component, and soy protein containing lower isoflavone concentrations would be warranted, especially in male subjects, where the plasma isoflavone concentrations are higher than in female subjects. The adverse effects of a high—soy isoflavone diet on insulin and glucose metabolism in male monkeys are of potential public health relevance, and studies are needed to determine the mechanisms involved and to optimize dietary isoflavone content for maximal health benefits.

**Acknowledgment/Conflict of Interest**

These studies were supported in part by grants HL45666 and HL79421 from the National Heart, Lung, and Blood Institute, and P40RR021380 from the National Center for Research Resources, all from the National Institutes of Health. The authors thank Ms Mary Jo Busa for editorial assistance.

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Modulation of peroxisome proliferator–activated receptor γ stability and transcriptional activity in adipocytes by resveratrol

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Abstract

The peroxisome proliferator–activated receptor (PPAR) γ is essential for the formation and function of adipocytes. It is also involved in regulating insulin sensitivity and is the functional target of the thiazolidinedione class of insulin-sensitizing drugs. Whereas thiazolidinediones activate PPARγ and decrease PPARγ protein levels, genetic models indicate that decreased expression of PPARγ is also associated with increased insulin sensitivity. In this study, we show that resveratrol modulates PPARγ protein levels in 3T3-L1 adipocytes via inhibition of PPARγ gene expression coupled with increased ubiquitin-proteasome–dependent degradation of PPARγ proteins. Resveratrol-mediated decreases in PPARγ expression are associated with repression of PPARγ transcriptional activity when assayed using a panel of PPARγ target genes in adipocytes. Finally, we demonstrate that resveratrol inhibits insulin-dependent changes in glucose uptake and glycogen levels and decreases insulin receptor substrate 1 and glucose transporter 4 protein levels, indicating that resveratrol represses insulin sensitivity in adipocytes. These results indicate that the resveratrol-mediated effects in adipocytes involve regulation of PPARγ expression and transcriptional activity along with decreased responsiveness to insulin.

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1. Introduction

A remarkable range of health benefits is ascribed to resveratrol, a bioactive plant polyphenol found in grapes, peanuts, and berries. Recent studies show that resveratrol treatment protects against diet-induced insulin resistance in rodents [1,2] and leads to decreased lipid accumulation in murine adipocytes [3]. Resveratrol is a potent activator of SIRT1 (silencing information regulator 1) [4], a histone deacetylase that mediates the effects of resveratrol in mice [1]. SIRT1 is also reported to inhibit the formation of adipocytes via repression of the peroxisome proliferator–activated receptor (PPAR) γ transcriptional activity [3].

Regulation of PPARγ activity in adipocytes provides a direct link between nutritional status, lipid metabolism, and adipocyte gene expression [5]. Adipose PPARγ is also required for the maintenance of insulin sensitivity [6], yet mice heterozygous for PPARγ deficiency remain more insulin sensitive than wild-type mice when fed a high-fat diet [7-9].

The reduced PPARγ gene expression in the PPARγ −/+ mice correlates with decreased PPARγ protein [10], suggesting that modulation of PPARγ activity and protein levels can play a role in regulating insulin sensitivity.

Activation of SIRT1 by resveratrol suggests a potential link between regulation of PPARγ activity, decreased PPARγ protein levels, and insulin sensitivity via resveratrol-mediated effects and points to a role for resveratrol in modulating insulin action associated with obesity and type 2 diabetes mellitus states. In the current study, we show that resveratrol treatment in 3T3-L1 adipocytes represses the endogenous gene expression of transcriptional targets of PPARγ such as aP2, Lpl, and Pepck. In addition, resveratrol decreases PPARγ gene expression while increasing targeting of PPARγ protein to the ubiquitin-proteasome system for degradation, a novel mechanism of resveratrol-mediated effects in adipocytes. We also show that resveratrol treatment in 3T3-L1 adipocytes reduces insulin sensitivity as measured by decreased insulin-dependent glucose uptake and glycogen content along with decreased protein content of insulin receptor substrate (IRS) 1 and glucose transporter (GLUT) 4. Thus, our data indicate that resveratrol may function as a nutritional regulator of PPARγ activity.
expression, and stability while also decreasing insulin sensitivity in adipocytes.

2. Materials and methods

2.1. Cell culture

Murine 3T3-L1 preadipocytes were plated and grown to 2 days postconfluence in Dulbecco modified Eagle medium high glucose with 10% bovine serum and penicillin/streptomycin. The cells were induced to differentiate using a standard induction cocktail of 3-isobutyl-1-methylxanthine, dexamethasone, and insulin as previously described [11]. The 3T3-L1 preadipocytes and fully differentiated adipocytes were maintained in a humidified chamber at 37°C.

2.2. Preparation of whole cell extracts

Cell monolayers were rinsed with phosphate-buffered saline (PBS) and harvested in a non-denaturing buffer as previously described [12]. Samples were extracted for 30 minutes on ice and centrifuged at 15,521 g at 4°C for 15 minutes. Supernatants containing whole cell extracts were analyzed for protein concentrations using a BCA kit (Pierce, Rockford, IL) according to the manufacturer’s instructions.

2.3. Thiazolidinedione and resveratrol treatment of 3T3-L1 adipocytes

Five micromoles per liter rosiglitazone (thiazolidinedione [TZD]) and 50 μmol/L resveratrol (Sigma-Aldrich, St Louis, MO) were added to fully differentiated 3T3-L1 adipocytes at the indicated times. Resveratrol was added in the indicated concentrations when glucose uptake was assayed in the presence of increasing concentrations of resveratrol. Dimethyl sulfoxide (DMSO) was used as a solvent for both rosiglitazone and resveratrol.

2.4. Gel electrophoresis and immunoblotting

Proteins were separated in polyacrylamide (National Diagnostics, Atlanta, GA) gels containing sodium dodecyl sulfate (SDS) according to Laemmli [13] and transferred to nitrocellulose. After transfer, the membrane was blocked in 4% nonfat dry milk suspended in PBS (pH 7.4) with 0.1% Tween 20 for 1 hour at room temperature. The membranes were incubated with mouse monoclonal anti-PPARγ or antibodies against IRS-1, IRS-2, P3K, AKT, phospho-AKT, PTP-1B, AMPKα1, AMPKα2, β-actin, anti-insulin receptor β subunit, GLUT1, and GLUT4 as indicated for 1 to 2 hours. After extensive washes with PBS (pH 7.4) with 0.1% Tween 20, the results were visualized with horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence (Pierce).

2.5. Real-time reverse transcriptase polymerase chain reaction

Total RNA was purified from cultured cells using TRI-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed via 2-step RT-PCR (High Capacity cDNA Archive Kit; Applied Biosystems, Foster City, CA) followed by PCR using TaqMan primer/probe pairs consisting of 2 sequence-specific PCR primers and a TaqMan assay-FAM dye-labeled MGB probe (Applied Biosystems, Taqman Gene Expression Assay) for each gene of interest. The genes of interest were fatty acid binding protein 4 (aP2), lipoprotein lipase (Lpl), and cytosolic phosphoxynolyptenase kinase (Pepck). The PCR was performed using the 7900 Real-Time PCR system (Applied Biosystems) under universal cycling conditions. All results were normalized to a cyclophilin B expression control and reported as the mean or the fold change relative to baseline ± standard deviation.

2.6. Ubiquitin conjugation assay

The 3T3-L1 adipocytes were preincubated with 10 μmol/L MGI32 and 1 μmol/L epoxomicin for 1 hour before adding 50 μmol/L resveratrol, 5.0 μmol/L rosiglitazone, or an equal volume of DMSO as a vehicle control. The cells were harvested after 30 minutes and lysed on ice in PBS containing 1% Triton X-100, 10 mmol/L N-ethylmaleimide, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μmol/L pepstatin, and 10 μmol/L leupeptin. Whole cell extracts were incubated with protein A–sepharose (RepliGen, Waltham, MA), and the unbound supernatant was collected for immunoprecipitation using a polyclonal anti-PPARγ followed by incubation with protein A–sepharose. The PPARγ–ubiquitin conjugates were detected by Western blotting using monoclonal anti-PPARγ and polyclonal anti-ubiquitin antibodies.

2.7. Determination of 2-[3H] deoxyglucose uptake

Fully differentiated 3T3-L1 adipocytes at day 6 to 7 post-induction were incubated in the presence of resveratrol, rosiglitazone, or DMSO for 6 hours. Four hours before measuring glucose uptake, the cells were serum-deprived in Dulbecco modified Eagle medium containing 6.25 mmol/L glucose and 0.3% bovine serum albumin. At the end of each treatment, 2-[3H] deoxyglucose uptake measurements were performed in triplicate; and the results were corrected for nonspecific uptake, which was measured in the presence of 5 μmol/L cytochalasin B [14]. The protein concentration of each lysate was determined using a BCA kit according to the manufacturer’s instructions.

2.8. Glycogen content

The adipocytes were treated with rosiglitazone (5 μmol/L) or resveratrol (50 μmol/L) for 15 hours, with serum derivation during the final 2 hours of treatment. The cells were then incubated with 30 mmol/L glucose in the absence or presence of 100 mmol/L insulin. At the end of 2 hours, the cells were washed 3 times with cold PBS (pH 7.4); and whole cell extracts were harvested in 200 μL of 0.2 mol/L sodium acetate.
acetate (pH 4.8), followed by sonication. After removal of 50-μL aliquots for protein concentration determination, glycogen content was measured according to the method of Gomez-Lechon et al [15].

3. Results

3.1. Resveratrol treatment increases proteasome-dependent PPARγ degradation and decreases PPARγ gene expression

Overexpression of SIRT1 is associated with decreased PPARγ protein levels in 3T3-L1 adipocytes [3], suggesting a link between activation of SIRT1 via resveratrol and regulation of PPARγ protein levels. To determine the effect of resveratrol on PPARγ protein levels, we assayed the steady-state levels of PPARγ proteins in fully differentiated 3T3-L1 adipocytes. As shown in Fig. 1A, a 6-hour treatment with either rosiglitazone or resveratrol decreases the steady-state levels of PPARγ proteins in adipocytes. We and others previously demonstrated that activation of PPARγ is linked to the ubiquitin-proteasome–dependent degradation of PPARγ [12,16]. Therefore, we asked if resveratrol-mediated decreases in PPARγ protein levels were also associated with direct targeting of PPARγ for ubiquitin conjugation and degradation by the proteasome. As shown in Fig. 1A, a 1-hour pretreatment of the adipocytes with the 20S proteasome inhibitors MG132 and epoxomicin partially reverses the effect of either rosiglitazone or resveratrol, but does not return PPARγ protein levels to those observed under control conditions in the presence of proteasome inhibition. The observed decreases in PPARγ protein levels are associated with increased targeting of PPARγ for ubiquitin-dependent degradation as shown by the increase in PPARγ-ubiquitin conjugate formation in the presence of resveratrol (Fig. 1B). The incomplete restoration of PPARγ protein levels in the presence of proteasome inhibition indicates that increased targeting for ubiquitin-proteasome–dependent degradation is insufficient to account for the resveratrol-mediated changes in PPARγ protein levels. As shown in Fig. 1C, both resveratrol and rosiglitazone also decrease Pparg gene expression.

3.2. Resveratrol treatment represses expression of endogenous genes regulated by PPARγ activity

The parallel effects of resveratrol and rosiglitazone on PPARγ protein degradation and gene expression prompted us to ask if resveratrol affects PPARγ transcriptional activity.

![Resveratrol decreases PPARγ gene and protein expression in 3T3-L1 adipocytes.](image-url)
To determine if resveratrol affects PPARγ activity, we used real-time RT-PCR to assay expression of PPARγ target genes in fully differentiated adipocytes rather than a luciferase-based assay of PPARγ transactivation. Previous studies have shown that resveratrol activates PPARγ in a variety of tissues [17-19], including macrophages [17] when measured using transactivation reporter assays. However, studies of other transcription factors such as Elk-1 and c-Fos [20] or control of T-cell receptor β expression [21] has demonstrated discrepancies between luciferase-based transactivation assays and regulation of endogenous targets of transcription factor activity. These studies point to the pitfalls of measuring gene expression outside the usual chromatin structure as is the case with luciferase reporter assays. This consideration is particularly relevant because resveratrol action involves activation of SIRT1, a histone deacetylase. To circumvent this problem, we chose a small set of genes in adipocytes that are involved in lipid metabolism and insulin sensitivity and whose expression is well described as PPARγ dependent: lipoprotein lipase (Lpl) [22,23], the fatty acid binding protein (aP2) [24], and the cytosolic phosphoenolpyruvate carboxykinase (Pepck) [25-27]. We assayed gene expression under control conditions or in the presence of resveratrol or rosiglitazone (TZD) for 6 or 15 hours. As shown in Fig. 2A, a 6-hour treatment with resveratrol represses aP2 and Lpl expression without affecting Pepck expression. Rosiglitazone (TZD) activation of PPARγ corresponds to increased expression of aP2 and Pepck in both cases with no increase in Lpl expression. However, a 15-hour treatment (Fig. 2B) with resveratrol shows repression of all 3 PPARγ target genes, whereas rosiglitazone treatment continues to be associated with increased expression of aP2 and Pepck.

3.3. Resveratrol decreases insulin-dependent glucose uptake in adipocytes

Modulation of PPARγ activity and gene expression is associated with improved insulin sensitivity when assayed as plasma glucose and insulin levels [7-9]. In addition, resveratrol treatment is associated with improved insulin sensitivity in murine models [1,2]. To determine if resveratrol affects insulin sensitivity in adipocytes, we measured the effect of resveratrol on glucose transport. The results in Fig. 3A demonstrate that resveratrol decreases insulin-stimulated glucose uptake in adipocytes when compared with control or TZD-treated conditions. In addition to triglyceride synthesis, insulin-stimulated glucose uptake in adipocytes leads to an increase in glycogen content via stimulation of glycogen synthase activity [28,29]. Therefore, we assayed the glycogen content of the 3T3-L1 adipocytes under control, rosiglitazone-treated, or resveratrol-treated conditions in the absence or presence of insulin. As shown in Fig. 3B, resveratrol treatment is associated with decreased glycogen content under basal and insulin-stimulated conditions. This is consistent with the observed resveratrol-mediated decreases in glucose uptake, indicating that resveratrol reduces insulin sensitivity in adipocytes.

In our experiments, we used resveratrol at a concentration previously shown to affect lipid accumulation in 3T3-L1 adipocytes [3]. In addition, resveratrol at 50 μmol/L has been shown to increase SIRT1 activity 3- to 4-fold [4]. To determine if the observed resveratrol-mediated decreases in insulin sensitivity were dose-related, we carried out glucose uptake assays in the presence of increasing concentrations of resveratrol (Fig. 3C). Insulin-stimulated glucose uptake is unaffected by treatment with resveratrol at 1 to 10 μmol/L, although basal levels of glucose uptake trend upward. Insulin-stimulated glucose uptake is inhibited in the presence of higher concentrations (30-100 μmol/L) of resveratrol.

3.4. Changes in insulin signaling pathway components in the presence of resveratrol

The resveratrol-mediated decreased glucose uptake suggests that resveratrol affects components of the insulin signaling pathway in adipocytes. As shown in Fig. 4, we assessed the content of proteins involved in the insulin signaling pathway. Resveratrol treatment decreased the basal levels of IRS-1 and the phosphorylated form of protein kinase B (AKT-P), and resulted in a small increase.
in insulin-stimulated AKT-P (Fig. 4A). In addition, total GLUT4 levels were decreased with resveratrol treatment (Fig. 4B). The observed changes in insulin sensitivity in the presence of resveratrol are consistent with decreased IRS-1 and GLUT4 protein levels, although contrary to the slight increase in insulin-stimulated AKT phosphorylation.

4. Discussion

Since PPARγ was identified as the functional receptor for the TZD class of insulin-sensitizing drugs [30], efforts to improve treatment of type 2 diabetes mellitus have included understanding the regulation of PPARγ in adipocytes. Although activation of PPARγ by the TZDs increases insulin sensitivity, studies of mice heterozygous for PPARγ show that reduced gene expression of wild-type PPARγ also improves insulin sensitivity [7,9]. The improvement in insulin sensitivity was observed with aging [8] and may include resistance to changes in insulin sensitivity that accompany a high-fat diet [7,8]. This is in contrast to the effect of a dominant negative PPARγ mutation (P465L) in leptin-deficient mice (P465L/ob) that results in insulin resistance in a setting of positive energy balance [31]. The wild-type PPARγ −/+ genetic model indicates that modulation of wild-type PPARγ expression could offer an alternative approach in the treatment of type 2 diabetes mellitus [32].

The present studies demonstrate that PPARγ transcriptional activity and protein levels are modulated in adipocytes by resveratrol, a bioactive plant polyphenol. The decreased levels of PPARγ proteins in 3T3-L1 adipocytes in response to resveratrol are mediated by decreased Pparg gene expression coupled with increased ubiquitin-proteasome-dependent degradation of PPARγ proteins, paralleling the effect of TZDs. Earlier evidence indicated that PPARγ is targeted for destruction via the ubiquitin-proteasome system under basal or activated conditions [14,16,33], supporting a model in which PPARγ degradation serves to limit PPARγ transcriptional activity. Therefore, downregulation of PPARγ in the presence of resveratrol describes a novel mechanism of action for resveratrol that is consistent with the overall scheme of limiting PPARγ activity via ubiquitin-proteasome–dependent degradation.

Resveratrol-mediated reductions in PPARγ gene expression and PPARγ proteins correlate with decreased cellular effects of insulin and insulin signaling proteins in adipocytes as assayed by decreases in protein content for IRS-1 and GLUT4 in addition to glucose uptake and glycogen content. Assessment of functional aspects of GLUT4 properties such as translocation to the plasma membrane was outside the scope of the current study. Although resveratrol treatment [2]
and reduction in PPARγ expression [7-9] are associated with generally improved insulin sensitivity in murine models, the observed effect of resveratrol in adipocytes is consistent with studies on longevity demonstrating that calorie restriction is associated with inhibition of insulin signaling [34]. In particular, selective loss of the insulin receptor in murine adipocytes (FIRCO mice) protects against developing age- and obesity-related insulin resistance [35]. The reduction in insulin sensitivity in adipocytes in response to resveratrol may mimic the overall insulin-sensitizing effects of calorie restriction, where the predominant feature is a loss of fat mass as a result of decreased lipid storage and increased lipolysis in adipocytes [36]. Clearly, any generalized improvement in insulin sensitivity coincident with decreased glucose uptake in adipocytes will involve the interaction of adipocytes with other tissues that are glucose responsive, such as skeletal muscle and the liver, as well as independent effects of resveratrol on those tissues [1,2]. Although the current studies are not designed to determine if the resveratrol-mediated changes in insulin sensitivity are due to down-regulation of PPARγ activity and expression in adipocytes, our results are consistent with a recent finding from Liao et al [37] showing that direct attenuation of PPARγ expression decreases glucose uptake in 3T3-L1 adipocytes. Thus, resveratrol may serve as a pharmacological tool to explore the effects of reducing PPARγ protein and gene expression in adipocytes.

Acknowledgment/Conflict of Interest

This work is funded in part by the National Institutes of Health Grant P50AT002776-01 from the National Center for Complementary and Alternative Medicine (NCCAM) and the Office of Dietary Supplements (ODS) and by the National Institute on Aging R03 AG025751 (to ZEF). The authors thank Dr Jeffrey Gimble for helpful discussions and critical reading of the manuscript.

References


Quercetin transiently increases energy expenditure but persistently decreases circulating markers of inflammation in C57BL/6J mice fed a high-fat diet

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Abstract

Quercetin, a polyphenolic compound and a major bioflavonoid in the human diet, has anti-inflammatory properties and has been postulated to enhance energy expenditure (EE). We sought to determine whether quercetin alters body weight, body composition, EE, and circulating markers of inflammation. At 6 weeks (W) of age, 2 cohorts of C57BL/6J mice (N = 80) were placed on one of 2 diets for 3W or 8W: (1) high fat (HF) (45% kcal fat) or (2) high fat + quercetin (HF + Q) (45% kcal fat + 0.8% quercetin). Quercetin concentrations in the diet and plasma were evaluated using mass spectrometry. Body weight, composition (nuclear magnetic resonance), and food consumption were measured weekly. Energy expenditure was measured by indirect calorimetry at 3 and 8W, and inflammatory markers were measured in plasma obtained at 8W. The presence of quercetin in the HF diet did not alter food consumption over time in the HF + Q group and did not differ from the HF group at any time point. However, circulating plasma quercetin concentrations declined between 3 and 8W. At 3W, EE was higher during both day and night phases ($P < .0001$) in the HF + Q group compared with the HF group; but this difference was not detected at 8W and did not translate into significant differences between the HF + Q and HF groups with respect to body weight or body composition. During the night phase, concentrations of the inflammatory markers (interferon-$\gamma$, interleukin-1$\alpha$, and interleukin-4) were significantly lower when compared with HF treatment group ($P < .05$). Dietary supplementation with quercetin produces transient (3W) increases in EE that are not detected after 8W on the diet. A corresponding decrease in circulating quercetin between 3 and 8W suggests that metabolic adaptation may have diminished the impact of quercetin’s early effect on EE and diminished its overall effect on nutrient partitioning and adiposity. However, quercetin at the levels provided was effective in reducing circulating markers of inflammation observed in animals on an HF diet at 8W.

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1. Introduction

It is estimated that more than 1 billion adults worldwide are overweight, and at least 300 million are considered obese. Major contributors to this epidemic across the world are secondary to sedentary lifestyles; consumption of high-fat, caloric-dense diets; and increased urbanization. Obesity predisposes one to development of type 2 diabetes mellitus and cardiovascular disease, and has also been considered a low-grade inflammatory disease [1]. Importantly, it has been well established that a reduction in body weight in the range of 5% to 10% can significantly slow the progression of these conditions [2]. Thus, although it is well established that caloric restriction and exercise greatly improve insulin resistance, the success of lifestyle intervention in long-term maintenance of weight loss is poor. Therefore, strategies to decrease weight by pharmacologic or nutritional supplementation represent a very attractive approach; and the flavonoids have been advanced as a potential botanical component of an effective obesity treatment regimen [3].
Table 1
Diet composition

<table>
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<td>L-Cystine</td>
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</table>

FD&C indicates foods, drugs and cosmetics.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a polyphenolic compound in the flavonol subgroup of flavonoids [4]. Although quercetin is the major bioflavonoid in the human diet [5], US consumption of dietary quercetin is only about 30 mg/d [6]. High concentrations are found in apples, tea, onions, and red wine [7]. Although absorption of quercetin occurs within 30 minutes of ingestion [8], tissue distribution of quercetin requires further evaluation.

The flavonols and quercetin have been proposed to increase cellular energy expenditure (EE) by elevating oxygen consumption in human skeletal myocytes. The mechanism is thought to involve up-regulation of type 2 deiodinase expression, which has been shown to increase EE [9] by increasing formation of triiodothyronine [10]. Although these data are promising, rigorous evaluation of well-characterized compounds in physiological models is needed to provide more convincing results. Specifically, a common problem with many of the studies evaluating quercetin is imprecise information regarding the amount of compound actually provided, leaving significant uncertainty as to the effective dose of quercetin required to achieve the desired response [11].

Many studies report that quercetin has potent anti-inflammatory properties [12]. Although the underlying mechanisms for this response are unclear, it has been suggested that quercetin’s anti-inflammatory properties may be due to its ability to decrease production of inflammatory cytokines [13]. Given the potential of quercetin to increase EE and mediate anti-inflammatory activities, the present study was designed to provide a comprehensive phenotypic evaluation of the therapeutic responses to a defined dose provided as a food admixture in the well-characterized C57BL/6J model of diet-induced obesity.

2. Methods

2.1. Animals

Male C57BL/6J mice (N = 80) (Jackson Laboratory, Bar Harbor, ME) were weaned onto a low-fat diet (10% kcal fat; Research Diets, New Brunswick, NJ) and, at 6 weeks (W) of age, were randomized and given free access to either high-fat diet (HF, 45% kcal fat, D12451) or high-fat diet formulated to contain 1.2% quercetin (HF + Q, D06081502) (Table 1). Energy expenditure was measured in one cohort (n = 8 per treatment) at 3 and 8W. Food consumption and body composition were measured in 2 cohorts (n = 8 per treatment per cohort) of mice that were killed at the zenith (midnight) or nadir (noon) of the metabolic cycle at the 3 and 8W time points. All mice were singly housed in shoebox cages with corncob bedding in controlled environmental conditions (22°C) on a 12-hour light/dark cycle. All experiments were reviewed and approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee.

2.2. Diets and food consumption

Research Diets formulated the HF + Q diet by cold processing to contain 1.2% (wt/wt) quercetin (Sigma Aldrich, St Louis, MO; ≥98% purity by high-performance liquid chromatography [LC], Q0125). To establish final quercetin concentration in the diet and assess its stability during storage, the HF + Q diet was evaluated using mass spectrometry (MS) at the beginning of the study and in a sample that was stored for 8W in the conditions described above. Briefly, 1-g samples of murine high-fat diet formulated with quercetin were powdered with a mortar and pestle and extracted with 5 mL of 95% ethanol. One-gram samples of murine high-fat diet without quercetin were spiked with 12 mg of quercetin and processed simultaneously. The solids were removed by centrifugation. The extract was dried with a SpeedVac (Thermo Scientific, Waltham, MA), resuspended in 1 mL of water, and partitioned 3 times with 1 mL of water.
ethyl acetate, dried, and resuspended in 500 μL of hexane. The water was then partitioned 3 times with 1 mL of water and partitioned once with hexanes (2 mL) to defat. The samples were then partitioned 3 times with 2 mL of ethyl acetate. The samples were then vortexed and placed back into the sonicator for 15 minutes at 50°C. The samples were then partitioned 3 times with 1 mL of ethyl acetate, dried, and resuspended in 500 μL ethanol for LC/MS analysis as described below.

Frozen blood plasma (200 μL) samples were thawed and immediately diluted with 200 μL water and 500 μL of 100 mmol/L sodium acetate buffer with a pH 5. β-Glucuronidase type HP-2 (20,014 U, Sigma) was then added, briefly vortexed, and incubated at 37°C for 30 minutes. After incubation, the samples were transferred to glass tubes with 1 mL of water and partitioned once with hexanes (2 mL) to defat. The samples were vortexed and sonicated at 50°C for 15 minutes and centrifuged at 5000 rpm for 6 minutes. The hexane fraction was then removed and discarded. The samples were then partitioned 3 times with 2 mL of ethyl acetate. The samples were then vortexed and placed back into the sonicator for 15 minutes at 50°C. The samples were then placed in the centrifuge and spun at the same speed and time. The ethyl acetate partitions were dried in a SpeedVac and resuspended in 125 μL of 95% ethanol in a sonicator at 50°C for 10 minutes. After centrifugation, the samples were analyzed with an LC/MS system as previously described [14].

2.4. Body weight and composition

Body weight and composition (nuclear magnetic resonance [NMR]; Bruker Minispec, Billerica, MA) were measured on a weekly basis in 2 cohorts of mice per treatment group (n = 8 mice per treatment per time point) for the duration of the study. Body composition was measured using a mouse NMR (Bruker Minispec) on a weekly basis. Fat mass (FM) was subtracted from total body weight to determine the fat-free mass (FFM). Percentage fat and percentage FFM were calculated by dividing FM or FFM by total body weight and multiplying by 100.

2.5. Energy expenditure

Body weight and body composition (mouse NMR, Bruker Minispec) were evaluated in a separate cohort (n = 8 mice per treatment per time point) and animals that were singly housed and given free access to their prescribed diet (specified above) and water in the metabolic chambers. After an overnight acclimation to the chambers, the volume of oxygen consumption (VO₂) and the CO₂ production were measured for 72 hours using indirect calorimetry and used to calculate respiratory exchange ratios (RERs) for each recording interval. Recording intervals were 60 seconds in duration, and one reading was completed on all animals every 45 minutes. Energy expenditure was calculated using VO₂ and RER (VO₂ × [3.815 + (1.23 × RER)] × 40.1868) and expressed as kilojoules per kilogram FFM per hour (Flowmax; Columbus Instruments, Columbus, OH).

2.6. Circulating inflammatory markers

The EDTA-treated blood samples were obtained by cardiac puncture from mice at the zenith and nadir of the metabolic cycle (n = 8 mice per treatment) at 8W. Circulating concentrations of inflammatory markers were measured in duplicate with 25 μL of plasma using an 8-plex (interleukin [IL] 1β, monocyte chemoattractant protein [MCP] 1, tumor necrosis factor [TNF] α, IL-1β, IL-2, IL-4, IL-10, and interferon [IFN] γ) Linco Research (St Charles, MO) murine multiplex kit on a LumineX 100 (Austin, TX) platform.

2.7. Statistical analysis

The data were analyzed with JMP statistical analysis software (SAS, Cary, NC), and results were expressed as means ± standard error. All measurements were evaluated using a 1-way analysis of variance (ANOVA) designed to examine the differences between the groups (HF and HF + Q) at the 3 and 8W time points, respectively. A Dunnett’s test was used post hoc as necessary. A P value < .05 was used to assess significance.

3. Results

3.1. Quercetin concentration and stability in the diet

Using MS, quercetin was not detected in the high-fat diet formulated without quercetin. In the high-fat diet formulated with quercetin, 67% of the 12 g/kg (1.2%) initially added to the diet was recovered by MS, resulting in an actual final concentration of 0.80 g quercetin per kilogram diet. Quercetin was also measured in samples of the HF + Q diet held in the diet storage area for the duration of the study. The quercetin concentration measured in these samples was 0.79%, which corresponds to a 1% decrease in quercetin during the 8W of storage.

3.2. Circulating quercetin concentration and food consumption

Because quercetin was administered as a food admixture and mice have a diurnal pattern of food consumption, quercetin was measured in blood samples taken in the middle of their nighttime and daytime cycles after 3 and 8W. Mice...
are nocturnal feeders and consume most of their 24-hour food consumption at night when they are awake. As expected, plasma quercetin concentration was higher in nighttime blood samples vs daytime blood samples at both 3 and 8W (Fig. 1). Plasma quercetin was 1.5-fold higher in samples taken at night vs those taken during the day at the 3W time point and 2-fold higher after 8W. If we take plasma quercetin averaged over night and day as a measure of overall treatment with quercetin, total plasma quercetin decreased by $\sim 80\%$ between 3 and 8W (Fig. 1). This observation indicates a significant decrease in effective dosing of the mice with quercetin between 3 and 8W, and examination of food consumption between the groups during this period provides no evidence for a decrease in consumption of the HF + Q diet as a basis for the decrease in circulating quercetin (Fig. 2). Food consumption over the 8W study averaged 23.41 ± 0.09 kcal/48 h (HF + Q) and 24.05 ± 0.12 kcal/48 h (HF) in the respective groups, and there was no evidence that food consumption changed over time in either group (Fig. 2). Collectively, these results show that the decrease in circulating quercetin in the HF + Q group between 3 and 8W is not due to a decrease in the consumption of the diet or a decrease in the stability of quercetin in the diet. These results are consistent with a change in quercetin pharmacokinetics between 3 and 8W, although the nature of the change remains to be established.

3.3. Change in body weight and composition over time

Mean body weights did not differ between the groups at the beginning, end, or at any week during the course of the study. The mice in both groups gained an average of 9 g of body weight during the 8W study (Fig. 3), and there was no evidence that the composition of the added weight differed between the groups (Fig. 4). For example, Fig. 4 shows that the relative adiposity (grams fat per gram body weight $\times 100$) of mice in both groups was $\sim 10\%$ at the beginning of the study and $\sim 20\%$ at the end of the study. Adiposity did not differ between the groups at either of these time points or at any time point between (Fig. 4). Given that food consumption also did not differ between the groups at any point, the results show collectively that quercetin altered neither postweaning growth nor the composition of that growth in C57BL/6J mice weaned onto a high-fat diet.

**Fig. 2.** Change in food consumption (kilocalories per day) over time in mice provided high-fat diets (45 kcal %) containing either vehicle (HF) or 0.8% quercetin (HF + Q). Food consumption, accounting for spillage, was measured over a 2-day period each week for 32 mice per diet from 0 to 3W and 16 mice per diet from 4 to 8W. Means were compared by 2-way ANOVA as described in Methods to test for diet-dependent differences in food consumption each week. Mean food consumption did not differ between the diets at any week during the study.

**Fig. 3.** Change in body weight over time in mice provided high-fat diets (45 kcal %) containing either vehicle (HF) or 0.8% quercetin (HF + Q). Body composition was determined each week by NMR as described in Methods, and FM was expressed as a percentage of body weight to measure relative adiposity. Means were compared by 2-way ANOVA to test for diet-dependent differences in adiposity at each week. Mean adiposity did not differ between the 2 groups at any week during the study.

**Fig. 4.** Change in FM as a percentage of body weight over time in mice provided high-fat diets (45 kcal %) containing either vehicle (HF) or 0.8% quercetin (HF + Q). Mean body weight did not differ between the groups at any week during the study.
3.4. Energy expenditure

Oxygen consumption and CO₂ production were measured by indirect calorimetry after 3 and 8W on the respective diets to test whether quercetin altered EE or respiratory quotient (RQ) in this study. Total EE expressed relative to lean mass was significantly elevated in the HF + Q group compared with the HF group at the 3W ($P < .0001$) but not at the 8W time point (Fig. 5A, B). The increase in EE detected at the 3W time point was evident not only in comparisons made during the day ($P < .001$), when mice normally sleep, but also in nighttime comparisons, when mice are awake, physically active, and consuming food ($P < .001$). This outcome is indicative of an overall increase in metabolic rate rather than an exaggerated thermogenic response to food. Furthermore, when physical activity was assessed by interruption of infrared beams, no evidence was found to support differences in voluntary activity as a basis for increased EE during the day (HF mean: 2234.72 ± 237.72 beam breaks; HF + Q mean: 2415.61 ± 207.03 beam breaks; $P = .57$) or night (HF mean: 24875.6 ± 1648.8 beam breaks; HF + Q mean: 22656.2 ± 1556.6 beam breaks; $P = .5699$, $P = .34$). Thus, at 3W, there was an increase in EE in HF + Q animals, but no change in food intake, voluntary activity, weight, body composition, or substrate selection. Examination of EE after 8W on the respective diets found no evidence of increased EE in the HF + Q group during the day or night, indicating that the early effect of quercetin at 3W did not persist for 8W (Fig. 5B).

Respiratory exchange ratio is an indicator of the composition of metabolic fuels being oxidized at any point in time, with RERs near 1 indicative of a sole reliance on carbohydrate as a fuel source and RERs near 0.7 indicative of fat as a fuel source. The RERs calculated from the respective groups provide no evidence to suggest that quercetin altered substrate selection at 3W (Fig. 5C), when EE differed between the groups (Fig. 5A), or at 8W (Fig. 5C), when no effect of quercetin on EE was evident (Fig. 5B). Collectively, these findings show that quercetin increased EE without altering substrate selection at 3W, but the effect on EE was no longer evident at 8W. Although the connection remains to be established, this loss of effect coincides with the significant decrease in circulating quercetin between 3 and 8W (Fig. 1).
3.5. Circulating markers of inflammation

Differences in inflammatory protein levels between the treatment groups were most significant during the night phase (Fig. 6) at 8W. More specifically, the HF + Q group was lower in INF-γ (P = 0.05), IL-1α (P = 0.0285), and IL-4 (P = 0.0464) (Fig. 6). At 8W, during the day, INF-γ trended to be lower in the HF + Q compared with the HF (P = 0.08) group. Interleukin 2 (P = 0.0250), MCP-1 (P = 0.0480), and TNF-α (P = 0.0195) at night were significantly higher compared with those during the day in the HF group.

4. Discussion

The promotion of the health benefits of quercetin has spurred increased interest in the scientific community [15] and the dietary supplements industry [6]. Specifically, a 21% reduction in cardiovascular disease mortality in humans has been reported for intakes of quercetin of greater than 4 mg/d [16]. Castilla et al [17] found that red grape juice, of which quercetin is the primary polyphenol, increased high-density lipoprotein concentrations and reduced low-density lipoprotein and oxidized low-density lipoprotein. Given the potential of quercetin to act as an anti-inflammatory agent, it is surprising that studies addressing the influence of quercetin on body weight, composition, and the corresponding alterations of inflammatory markers are limited.

Our data clearly demonstrate that continuous quercetin ingestion increased EE when measured after 3W but not after 8W. The failure to maintain increases in EE and reductions in plasma quercetin concentrations at 8W suggests a metabolic adaptation or change in pharmacokinetics to the quercetin supplementation. Despite these observations, quercetin remained effective in reducing the inflammation observed in animals on an HF diet at 8W. Although it remains to be established, these findings are consistent with the possibility that the change in circulating quercetin between 3 and 8W was sufficient to drop the quercetin dose below the threshold for effects on EE. It is also possible that the effective dose needed for reducing inflammatory markers is below the dose needed for effects on EE. These questions will need to be addressed directly in future studies.

An important question raised by our findings is the apparent disconnect between the effect of quercetin on EE (Fig. 5A) and the lack of an effect on growth or body composition (Figs. 3 and 4). We expected that the increase in EE at 3W would translate into reduced fat deposition, particularly because food consumption did not differ between the groups. However, if the effect of quercetin on EE was short-lived and lost earlier than the 8W time point documented here, it is unlikely that the modest effect of quercetin on EE noted at 3W would be sufficient to impact overall energy balance and fat deposition. Our body composition data from the 3W time point (Fig. 4) support this interpretation, but it will be important in future studies to determine the time course and duration of the effect of quercetin on EE.

We found that consumption of an HF diet supplemented with 0.8% quercetin for 3W increased EE relative to HF controls. The higher EE detected at 3W did not translate into significant differences in body weight (Fig. 3) or adiposity (Fig. 4). The lack of a quercetin-mediated shift in substrate utilization (Fig. 5C) suggests that the magnitude of the difference in EE between the groups was not significant enough to influence body composition. A few other studies support our findings. For example, a 0.5% quercetin diet fed to 1-month-old Swiss mice for 28 days did not result in changes in body weight compared with control animals [18]. Similar results were seen in rats fed high-fat, high-sucrose diets containing 0.02%, 0.2%, and 0.07% quercetin fed ad libitum for 4W [19].

Surprisingly, after 8W, there were no significant differences between EE in the HF and HF + Q groups (Fig. 5B.). In addition, blood plasma concentrations of quercetin were higher at the 3W time point than at the 8W time point for both day and night measures. Although circulating levels of quercetin were higher at night, which coincides with the nocturnal feeding habits of the mice and the long half-life (~24 hours) of quercetin [20], the diurnal variation at 8W is greater, with significant differences noted at the 8W time point and not at 3W (Fig. 1). Together, these results suggest a metabolic adaptation to the diet such that the mice were able to quickly remove the quercetin from their system after 8W. Little is known about the adaptation to quercetin in the diet, and further exploration would be essential to understanding the benefits and/or risks associated with this botanical compound.

Given our discrepancy between 3 and 8W EE measurements, absolute levels, and diurnal changes in circulating quercetin concentration, it is important to note that diet composition is linked to quercetin absorption. A high-fat diet was chosen not only because it induces obesity in C57BL/6J mice, but also because quercetin absorption is greater and elimination is delayed with high-fat diets in other animal (porcine) models [8]. Specifically, increasing the fat content in quercetin-enriched diets from 3% to 17% resulted in a 50% increase in total bioavailability of quercetin in pigs [8]. In addition, the method of delivery has been implicated as a major factor in the effectiveness of the treatment; and it serves as a confounding variable when comparing our results to other studies. Injection [21], gavage [22], and addition of quercetin to food [22] or drinking water [23] are most commonly used. In a recent study, 5W-old spontaneously hypertensive rats (SHR) were provided 1.5 g quercetin per kilogram of diet for 5W or 11W [22]. Although there was an absence of a delayed progression of the cardiovascular morbidities that occur in SHR rats in those animals on the dietary intervention, 15W-old SHR when gavaged once daily with quercetin (10 mg/kg) for 4 days were observed to have a decreased arterial pressure when compared with the untreated control groups [22].
We observed a quercetin-mediated decrease in circulating markers of inflammation in a physiological model that were consistent with previous studies that have addressed quercetin’s action in cell culture models [24,25]. Ishikawa et al [25] demonstrated the ability of quercetin to suppress nuclear factor–κB in glomerular cells, thus inhibiting IL-1–triggered MCP-1 expression. Quercetin has also been shown to reduce macrophage expression of TNF-α, IL-1β, and IL-6 in a dose-dependent manner through an inhibition of lipopolysaccharide-induced expression of these proinflammatory cytokines [26]. Quercetin has also been associated with an inhibition of protein kinase C translocation and the activation of extracellular signal–regulated kinases and c-Jun NH2-terminal kinase, but did not influence the activation of the p38 mitogen–activated protein kinase (MAPK) pathway in human cultured mast cells. In other cell lines, however, MAPK pathway activation is inhibited by quercetin. For example, MAPK activation in human epidermal carcinoma cells was inhibited by quercetin (30 μmol/L) [27].

Once absorbed, quercetin is usually present in glycosylated forms, such as quercitrin (3-rhamnosylquercetin) and rutin. Previous in vitro experiments have shown that quercetin is more effective, when compared with quercitrin, in the down-regulation of the inflammatory responses [28]. This down-regulation was shown to occur via the nuclear factor–κB pathway without modification of c-Jun N-terminal kinase activity (both in vitro and in vivo) resulting in lower levels of cytokine and inducible nitric oxide synthase expression in a rat model of colitis. A more extensive exploration of the mechanisms associated with quercetin-mediated reductions in inflammation in physiological models is a promising area for future research.

The present study derives its novelty in part from rigorous attention to establishing the identity and purity of the quercetin used to formulate the diet, establishing the concentration and stability of quercetin in the diet, carefully measuring food consumption, and assessing a composite of the pharmacokinetics of the compound by measuring concentrations of quercetin in the blood at the peak and nadir of the metabolic cycle. Collectively, these aspects of the study provide a sound foundation for interpreting the physiological responses of the mice to the dose of quercetin administered in this work. The diet was originally formulated to contain 1.2% quercetin by weight, which translates to a dose of ∼30 mg/d per mouse based on an average food consumption of 2.5 g/d. However, upon analysis, the HF + Q diet actually contained 0.8% quercetin, which translates to a daily dose of 20 mg per mouse or 0.8 mg/d per gram of body weight for the weight range of mice in our study. Previous studies have used a variety of doses ranging from 50 μg/d per mouse [23] to 10 mg quercetin per kilogram of body weight [29] to as high as 5000 mg per kilogram of body weight per day [30]. Quercetin administered at 10%, 4%, and 1% in a pellet diet was not carcinogenic; and when 1% croton oil was administered after 1% quercetin, there were no increases in tumor incidence in Chinese golden hamsters [31]. In addition, when oral doses of 1 to 1000 mg of quercetin per kilogram of body weight were given to male mice, there were no mutagenic effects, as defined by the micronucleus test or the Salmonella tester strain TA 98 host-mediated assay [32]. The maximum tolerable dose of quercetin was determined to be 2500 mg/kg twice per day through intraperitoneal injection. At this dose, as well as at 1250 and 625 mg/kg twice per day, DNA damage was detected with alkaline single-cell gel electrophoresis; however, because of the high doses, it was suggested that clastogenic effects due to consumption would be unlikely [30]. Doses of quercetin in human intervention studies have ranged from 21 to 1000 mg/d, with no adverse side effects [7].

In summary, this study provides novel insights into potential metabolic actions of dietary quercetin. A high-fat diet (45 kcal %) supplemented with 0.8% quercetin produced a transient increase in EE after 3W on the diet that was not evident after 8W. A corresponding decrease in plasma quercetin without any decrease in food intake suggests that adaptive changes in the pharmacokinetics of quercetin may have diminished its biological efficacy. However, the dose of quercetin provided in this study produced a significant sustained decrease in several circulating markers of inflammation at 8W. These changes occurred in the absence of any reduction in adiposity by quercetin, suggesting a potential use of this compound in the chronic low-level inflammatory state associated with obesity. A more in-depth examination of quercetin and inflammation as it relates to disease development is necessary.

Acknowledgment/Conflict of Interest

This work was supported by the National Center for Complementary and Alternative Medicine (NCCAM) and the Office of Dietary Supplements (ODS) (NIH Grant P50AT002776-01), and by support from NIH CNRU P30 DK072476 and P20 RR021945 (TWG). The authors thank Michael Pellizzon, PhD (Research Diets), for his expertise in the quercetin diet formulation and Jamie Tuminello and Jennifer Rood for assistance with the multiplex cytokine analysis.

References

Effect of Shilianhua extract and its fractions on body weight of obese mice
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Abstract

Five commercial botanical products (Shilianhua [SLH] tablets, Shiu Huo pills, Fenulyn, Bitter Melon, and Glucose Metabolic Support), available in the US market, with reported claims for regulation of metabolism were screened for their effect on body weight gain in high-fat diet-induced obese mice. Pilot results suggested that SLH tablets attenuated body weight gain, whereas Shiu Huo pills and Fenulyn tended to promote weight gain in the mice on the high-fat diet. To investigate the bioactive components in the SLH tablet, the wild SLH plant (Sinocrassula indica Berge) was collected from China and used to make a variety of extracts including aqueous extract, ethanol extract (SLH-E), and subfraction F100. In the study of metabolic activities, the extracts were administrated through food intake by incorporating them into the diet. A rigorous evaluation of the extracts on body weight was conducted in 2 animal models. The aqueous extract and SLH-E were tested in dietary obese mice, while F100 together with SLH-E was tested in KK-Ay mice, a genetic diabetic model. In the 12- to 16-week study, body weight was not significantly altered by the SLH extracts in the 2 animal models. The results suggest that neither the total extract nor the purified components from the SLH plant have a clear effect in the regulation of body weight. The weight reduction observed with the over-the-counter SLH tablet in the pilot studies may be secondary to other components in the tablet, but not from the SLH extract.  

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1. Introduction

Many over-the-counter (OTC) supplements have been marketed as being effective to promote glucose metabolism in humans, and consumption of dietary supplements by the general public has increased in the United States to facilitate the management of blood glucose [1]. Among these dietary supplements, botanical extracts are extremely popular components and have been promoted to enhance the therapeutic activities and reduce the adverse effects of synthetic drugs. However, the clinical efficacy and mechanism of action of many botanicals have not been well characterized. In this study, we evaluated the weight regulation activities of several botanical products that are marketed in the United States and make claims for metabolic regulation. Reduction of body weight is known to decrease blood glucose through improvement of insulin sensitivity. Five commercial products, based on reported claims, were chosen for evaluation in this study. Specifically, they were Shilianhua (SLH) tablets, Shiu Huo pills (SHP) (Chinese Medicine United Pharmaceutical Factory, Guangzhou City, China), Fenulyn (Fen) (Princess Lifestyle LLC, Baldwin Park, CA), Bitter Melon (BM) (Nature’s Herbs, American Fork, UT), and Glucose Metabolic Support (GMS) (Now Foods, Bloomingdale, IL). The efficacies of these products in the management of body weight were tested in the dietary obese mice by oral administration.

The commercial SLH tablet contains several botanical products including SLH (Sinocrassula Berger), spirulina, Lycium berries, soy, fiber, guar gum, et al. The SLH (Sinocrassula indica) is also patented to reduce blood glucose in the United States, Japan, and China. Shilianhua is the Chinese name for houseleek, which is widely distributed in the world. In addition, it is consumed as tea in Taiwan and Japan. Shilianhua is the Chinese name for houseleek, which is widely distributed in the world. In addition, it is consumed as tea in Taiwan and Japan. To study bioactivities of the SLH plant, we purified extracts from the wild S indica and examined their efficacy in the regulation of body weight in obese mice. The SLH extract was also divided into 4 subfractions, and one subfraction (F100) was tested in mice for weight regulation. The result suggests that the SLH extracts have no activities in the regulation of body weight.
2. Materials and methods

2.1. Mouse models and treatment

Dietary obesity was generated in the male C57BL/6J mice with a high-fat diet (HFD) as described elsewhere [2]. Male C57BL/6J mice and KK-Ay (KK.Cg-Ay mutant) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed singly in the study. The C57BL/6J mice were fed on an HFD (58% calories as fat, D12331; Research Diets, New Brunswick, NJ) at 5 weeks of age to induce obesity. The KK-Ay mice were fed a defined low-fat diet (D12329, Research Diets) throughout the experiment. The control group was fed the defined diet, and the treatment group was fed the same diet containing one of following products: SLH tablets (5.2 mg/[kg d]), SHP (100 mg/[kg d]), Fen (150 mg/[kg d]), BM (150 mg/[kg d]), and GMS (200 mg/[kg d]). In addition, animals had diets supplemented with SLH fractions (see preparation and purification below) consisting of SLH aqueous extract (SLH-A; 0.4% wt/wt), SLH ethanol extract (SLH-E; 1% wt/wt), or F100 at 0.05% (wt/wt). Body weight was measured weekly. Food intake was measured twice for 1-week intervals during weeks 3 and 6.

2.2. Preparation of the SLH-A

The photographs of fresh and dried SLH plants (S indica) are shown in Fig. 1A. The extract of SLH used in this study was prepared from the aerial parts of wild SLH plant. The plant was collected from the vast arid and mountainous areas in Guizhou province, southwestern China, where SLH has been used as a medicinal herb by the local residents for hundreds of years. The SLH sample was certified by a taxonomist at the Institute of Medicinal Plant Development, a Chinese authority in the identification and authentication of traditional Chinese herbs and medicinal plants. The fresh aerial part of SLH was air-dried under shade to reduce moisture content to approximately 8% wt/wt. The dry material was ground into 6-mm or smaller pieces. The grounded material was first soaked in deionized water at 1:8 wt/vol ratio for 60 minutes at room temperature and then extracted twice at 50°C in a rotary extractor for 6 hours. The water-soluble extract was separated from the solids (structural components of fibers, cellulose, semicellulose, debris of cells) by first centrifuging at 3500 rpm with an Allegra 6KR Centrifuge (Beckman Coulter, Palo Alto, CA) and then filtering with Whatman 4 paper. The liquid product was then concentrated in a rotary evaporator of 20-L capacity (Rotavapor R-220; Buchi, Flawil, Switzerland) and followed by freeze drying (Labconco, Kansas City, MO) into an aqueous extract powder (SLH-A) that accounted for 29.8% wt/wt of the raw herb.

2.3. Preparation of the SLH-E

The aqueous extract (about 1.3 kg) was further fractionated to yield a concentrated SLH extract. It was dissolved
into 15 L of deionized water, loaded into a 15-kg macroporous adsorbent polymer resin column (L493; Sigma Chemical, St Louis, MO), washed with 5 L of water, and then eluted with 20 L 95% ethanol, which was evaporated to obtain SLH-E.

2.4. Preparation of subfractions of SLH

The ethanol in 95% ethanol elutes was removed by evaporation under reduced pressure. The SLH was fractionated using a high-performance liquid chromatography (HPLC) system with C18 column (Sigma Chemical) that was eluted with water, 20% MeOH, 50% MeOH, and 100% MeOH in a sequence to obtain 4 subfractions, that is, F00, F20, F50, and F100. F100 was found to be the most active subfraction in the inhibition of inflammatory responses (data not shown) and was therefore selected as the subfraction for in vivo assessment on body weight gain along with the SLH-E and SLH-A extracts.

2.5. Characterization of SLH-E and subfractions

Chemical fingerprint of the SLH extracts was developed using a Waters (Milford, MA) HPLC system consisting of a Waters Delta 600 pump, a Waters 717 plus autosampler, and a Waters 2996 Photodiode Array Detector (190-800 nm) (Fig. 1B). The system is controlled by computer, and the data were analyzed with the Empower software system (Waters). The mobile phase consisted of HPLC-grade methanol and water run through a gradient elution from 3:97 (MeOH/H2O) to 10:90 (MeOH/H2O) for the first 6 minutes, followed by a gradient elution to 80:20 (MeOH/H2O) for 14 minutes and gradient elution to 100:0 (MeOH/H2O) for 5 minutes; kept for 5 minutes; and then equilibrated to 3:97 for 15 minutes before the next sample was injected.

2.6. Statistical analysis

In the bar figures, mean value and standard error of multiple data points or samples were used to represent the final result. Student t test or 1-way analysis of variance was used in the statistical analysis of the data, with a significance level of \( P < 0.05 \).

3. Results

3.1. Commercial SLH tablet attenuates diet-induced weight gain

The effects of 5 commercial products on body weight were investigated in dietary obese mice. The products were administrated through food intake by incorporating them into the HFD. Among the 5 products, the SLH tablets reduced gain in body weight on HFD (Fig. 2A, B). At the end of 12 weeks, the body weight was 38 g in the chow diet group (lean control) and 43 g in the HFD group (13% increase, \( P < 0.05 \)). With SLH supplementation, the body

![Image](image-url)

Fig. 2. Regulation of body weight by commercial botanical products. Five commercial botanical products including SLH, SHP, Fen, BM, and GMS were tested in the prevention of dietary obesity in mice fed an HFD. A, The SLH tablets reduced gain in body weight significantly when compared with the other commercial products. The body weight was examined at the end of 12 weeks on HFD. B, Picture of mice at 12 weeks on HFD and SLH tablets. C, Time course of body weight gain on HFD and SLH tablets. D, Food intake (grams) per body weight (grams) over 4 weeks from 12 to 16 weeks on HFD. Each point presents mean ± SE (n = 11). Compared with HFD control: *\( P < 0.01 \).
weight was 38 g in mice on HFD, which was identical to that of the chow diet group (Fig. 2C). With SHP or Fen supplementation, the body weight was 5% ($P < .05$) higher than that of the HFD group. These data suggest that SLH tablet attenuated body weight gain, whereas the SHP and Fen tablets promoted body weight gain in mice on HFD. This antiobesity effect of SLH was not a result of alteration in food intake because there was no significant difference in the food intake in the control (HFD) and experimental (SLH) groups (Fig. 2D). This pilot study suggested that the commercial SLH tablet may contain bioactive compounds that attenuates HFD-induced obesity in mice.

3.2. Chemical fingerprints of SLH extracts

Because the major component of the commercial SLH tablet is the extract of the Shilianhua plant, we made an effort to study the bioactivity in the plant $S$ indica. A variety of extracts was isolated from the plant, and their chemical fingerprints were analyzed with HPLC. Nine major components were observed in the chromatographic fingerprinting profile of SLH-E (Fig. 1B). The SLH-E was fractionated into 4 fractions in HPLC on the basis of polarity of components. These were identified as F00, F20, F50, and F100. The fingerprint profile suggests that components 1, 2, 4, and 5 were located in the F00 fraction; components 3, 5, 6, and 8 were included in the fraction F20; components 8 and 9 were contained in the fraction F50; and the least polar components were retained in the fraction F100. F100 was used as an organic extract of SLH because it was collected through elution with 100% MeOH.

3.3. Purified SLH extracts had no effects on body weight of mice

The purified SLH extracts were studied in the mouse model of obesity in identical experiments as assessed with the commercial products. First, SLH-A was tested in the dietary obese mouse model through oral administration (dietary supplementation). The dosage of extract was 0.4% (wt/wt) in the diet, which is equivalent to 500 to 700 mg/kg body weight per day. Both in the control and in the SLH-A–treated mice, body weight was increased in the mice in a time-dependent manner on HFD. No difference was observed between the SLH group and control group in body weight (Fig. 3A). Because SLH-A had no obvious actions in the dietary obese mouse model, we tested SLH-E in the HFD mice. The dose of SLH-E was increased to 1% (wt/wt) in the diet. No change in body weight was observed for SLH-E (Fig. 3B). Finally, SLH-E was also tested in KK-Ay diabetic mice that develop hyperglycemia and insulin resistance on low-fat diet. F100, a subfraction of SLH-E, was also tested in the study. The SLH-E and F100 were administrated orally through diet supplementation at 2% and 0.05% wt/wt in the low-fat diet, respectively. In the study, body weight was not significantly changed by SLH-E or F100 compared with the control mice (Fig. 3C), suggesting that the extracts of SLH were unable to regulate body weight in the diabetic KK-Ay mice.

4. Discussion

This study represents a very rigorous assessment of OTC supplements in the regulation of body weight. In this study, the commercial SLH tablet prevented body weight gain, whereas SHP and Fen promoted body weight gain in the dietary obese mice. However, when the major component of the SLH tablet was tested in the same animal model, no significant activity was observed in the regulation of body weight. Thus, this work strongly suggests that the SLH extract does not have an effect to regulate body weight.

The plant SLH is a shrub in the Crassulaceae family that grows in the southwestern part of China including Yunnan,
Guangxi, and Guizhou provinces. It had been used as an herb for hundreds of years in southwest China, and SLH is also patented to reduce blood glucose in the United States, Japan, and China. As we have described, when the 5 commercial products we tested were compared, only the SLH tablet was found to have a potent effect on regulation of metabolism. Thus, to identify the bioactive components for the activity for this commercial product, the SLH plant was obtained and used to make a variety of extracts including SLH-A, SLH-E, F00, F20, F50, and F100. Among them, the aqueous extract, that is, SLH-A, was the crude extract that contained all bioactive components except the fibers, cellulose, and debris of the cells that are felt not to be bioactive. The ethanol extract, that is, SLH-E, is a 95% ethanol extract of the plant and much more concentrated than SLH-A when assessing activity of inhibition of inflammatory response (data not shown). Compared with SLH-A, SLH-E contains more small-molecule compounds and fewer components with large molecules like polysaccharides and peptides, which are suggested to be inactivated in the gut after oral administration. In the subfractions of SLH, F100 was the organic extract with the lowest polarity in the SLH-E. F100 contains small molecules, such as saponin and alkaloid, which are water insoluble and with bioactivity in general. F100 is the most active subfraction in the inhibition of inflammation response (data not shown). In this study, SLH-A, SLH-E, and F100 were tested in dietary obese mice and KK-Ay mice, which are models for obesity and type 2 diabetes mellitus. However, no antiobesity effect was observed for these SLH extracts in the animal models. The results indicate that SLH has no bioactivities in the regulation of body weight. If the SLH plant does not have an activity in the regulation of body weight, what was responsible for the weight control activity of the SLH tablet in the pilot studies? First, the bioactivity of SLH tablet may be due to components other than SLH extracts. In addition to the SLH plant extract, the SLH tablet also contains products from 4 other plants, such as Spirulina maxima, Lycium berries, soy fiber, and guar gum. Spirulina was reported to reduce glucose and alleviate dyslipidemia and fatty liver [3-5]. Berry of Lyceum barbarum is a popular traditional Chinese herb that is used to treat aging-related disease. Berry of Lyceum barbarum was reported to have hypoglycemic, hypolipidemic, and antioxidiant effects in both type 1 and type 2 diabetes mellitus in animal models [6-9] Soy fiber is able to decrease postprandial blood glucose by regulation of glucagon, pancreatic poly-peptide, and somatostatin secretion [10,11]. Guar gum, a well-established water-soluble fiber, is known to reduce hypercholesterolemia, hyperglycemia, and obesity [12-14]. The combination of these botanical products in the SLH tablet may have constituted the antiobesity activity. However, to definitively state that the effect is from these extracts, the individual extracts also will have to be rigorously tested. In addition, it could also be argued that other components, for example, pharmacological agents, may have been involved. Over-the-counter agents are not regulated in the same fashion and do not have the same quality control standards as demanded by the Food and Drug Administration for prescription drugs. Thus, the claims made for products rarely are ever validated in placebo-controlled trials.

In summary, we conclude that the SLH plant as a total extract, or when separated into specific bioactive fractions, has no effects on regulation of body weight. The effect of weight reduction, if any, in the SLH tablet is clearly not due to SLH extract. As such, the effect of the commercial tablet on body weight with repeated testing was inconsistent. Thus, no scientific support exists for the role of SLH in the regulation of weight gain in our studies.

Acknowledgment/Conflict of Interest

Supported by NIH Grant P50AT002776-01 from the National Center for Complementary and Alternative Medicine (NCCAM) and Office of Dietary Supplements (ODS) and DK68036 to Ye J.

References

Gene expression microarray analysis of the effects of grape anthocyanins in mice: a test of a hypothesis-generating paradigm


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Abstract

The mechanism(s) through which fruits, vegetables, and whole grains favorably affect health is not well established. Using an anthocyanin-rich grape as a model, we examined the ability of an agnostic analytical approach using gene expression microarrays to generate novel testable hypotheses regarding the mechanisms of action of potentially healthful foods and food components. C57BL/6 mice were divided into 2 groups and fed a proatherogenic diet with or without a semipurified anthocyanin extract (70% anthocyanins) incorporated at a level of 0.1 mg/mL into the drinking water. After 6 weeks, compared with control mice, mice supplemented with anthocyanins tended to gain more weight and have increased adipose tissue mass, although these effects did not achieve statistical significance. Anthocyanin-supplemented mice had significantly reduced relative liver weights and heart weights. Serum lipids and inflammatory cytokines were not different between the groups. Gene expression microarray analysis of the liver and skeletal muscle identified a number of molecular pathways significantly affected by anthocyanin treatment. Two distinct clusters emerged. The first cluster included down-regulated pathways in both muscle and liver involving cellular defense, whereas the second included hepatic genes involved in energy metabolism. From these data, 3 hypotheses were developed for future investigation.

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1. Introduction

Increased consumption of fruits, vegetables, and whole grains and lower intakes of foods high in saturated fat are recommended by public agencies [1,2] largely because of supporting epidemiologic data. For saturated fat, the mechanism underlying the link between high intakes and disease (specifically, cardiovascular disease) is well established. Abundant evidence exists demonstrating an adverse effect of saturated fat on low-density lipoprotein cholesterol levels [3], a well-established risk factor for cardiovascular disease. However, the mechanism(s) through which fruits, vegetables, and whole grains favorably affect health is substantially less well established. We have previously suggested [4] that an agnostic approach (eg, without prior belief as to mechanism of action) using gene expression microarrays, proteomics, and metabolomic analytical methods could be used to generate novel testable hypotheses regarding the mechanisms of action of potentially healthful foods and food components.

In the present study, we apply gene expression microarray analysis to the investigation of the potential health benefits of an anthocyanin-rich grape extract (ACN-GE) as a partial test of this hypothesis-generating paradigm. Anthocyanins are polyphenolic compounds that provide color in berries such as grapes, blueberries, strawberries, and blackberries. Consumption in the United States is estimated at 12.5 mg/d [5]. Unlike other polyphenols, glycosides of anthocyanins are absorbed intact [6,7], suggestive of a potential unique role among polyphenols in human health. Previous work has focused on antioxidant and anti-inflammatory properties in relation to cardiovascular disease and maintenance of brain function with aging [8-13]. There is also evidence that anthocyanins may have
anticarcinogenic, antiobesity, and antidiabetic effects as well [14-16]. The availability of prior data regarding the potential health effects of anthocyanins provides an opportunity to validate our approach through corroboration of hypotheses generated from our analyses with existing published hypotheses.

2. Methods

2.1. Anthocyanin-rich extract preparation

An ACN-GE was prepared from the highly pigmented wine grape A-1575. The extracts were prepared by solid-phase extraction using Amberlite XAD-7 (Sigma-Aldrich, St Louis, MO) resin. The final extract contained 67% anthocyanins (Table 1) as assessed by high-performance liquid chromatography analysis and was exceptionally rich in malvidin glucosides, with moderately high levels of petunidin and delphinidin glucosides. High-performance liquid chromatography analysis additionally confirmed the extract to be free of free sugars and organic acids.

2.2. Animals and diets

Twenty 5-week-old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were maintained at constant temperature (22°C-24°C) under an automated lighting with a 12-hour/12-hour light/dark cycle throughout the experiment. The mice were divided into 2 groups of 10 and fed for 6 weeks a proatherogenic diet (D01022601; Research Diets, New Brunswick, NJ) to increase oxidative stress. The diet provided 39.9% of energy as fat and 1.5 g/kg cholesterol (Table 2). Diets were provided ad libitum for the duration of the study.

A 10-mg/mL stock solution of the ACN-GE was prepared in ethanol. The ACN-GE stock solution was added to the drinking water of one group of mice (ACN group) to provide a final concentration of 0.1 mg/mL ACN-GE and 1% ethanol mice. The control group received drinking water with added ethanol alone. The ACN-GE-supplemented drinking water was provided in brown water bottles and changed every other day. Preliminary studies demonstrated that, under these conditions, the anthocyanin preparations remained stable. Water intake was monitored and did not differ between groups. The experimental design was approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee.

2.3. Collection of serum and tissues

Two days before the end of the experiment, body composition was determined by nuclear magnetic resonance (model mq10 NMR analyzer; Brucker, Milton, Ontario, Canada). At the end of the feeding period, the mice were fasted for 4 hours before blood was obtained by cardiac puncture under anesthesia. The liver, heart, kidney, spleen, adipose tissue depots (subcutaneous, retroperitoneal, epididymal, brown), and sample of thigh skeletal muscle were harvested, weighed, and flash frozen in liquid nitrogen. Serum was collected by centrifugation. All tissue and serum samples were stored at −70°C until used for assays.

2.4. Measurement of serum glucose, triglyceride, cholesterol, hormones, and cytokines

Serum glucose, triglycerides, and cholesterol were measured using commercially available kits. Serum cytokines were measured by multiplexed immunobeads (Luminex, Austin, TX) with reagents purchased from LINCO (LINCOplex; Millipore, St Charles, MO).

2.5. RNA preparation

Ribonucleic acid was isolated from liver and skeletal muscle using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s protocol. Potential impurities were removed (RNaseasy Mini Kit; Qiagen, Valencia, CA), and the quality of RNA was assessed using a 1.5% agarose gel stained with ethidium bromide.

2.6. Gene expression analysis

Microarrays were prepared by printing oligonucleotides (mouse library; QIAGEN Operon, Alameda, CA) suspended
in 45% (vol/vol) dimethyl sulfoxide onto polylysine-coated, glass microscope slides using the GeneMachines OmniGrid microarrayer (San Carlos, CA). The mouse oligonucleotide library consists of 70mers that represent more than 13,000 well-characterized genes.

Gene-expression microarray analysis was performed using the MICROMAX TSA Labeling and Detection Kit protocol (PerkinElmer Life Sciences, Boston, MA). Samples from each treatment were pooled to yield a total of 4 to 6 μg of RNA per biotin (B) or fluorescein (F) label. A total of 6 slides were used per experiment with 3 for forward labeling (group 1 is B; group 2 is F) and 3 for reverse labeling (group 1 is F; group 2 is B). Slides were scanned using the ScanArray 5000 (Packard BioChip Technologies, Billerica, MA), and the data were normalized [17].

2.7. Statistical analysis

All phenotypic data are presented as mean ± SEM. Differences between means were assessed by Student t test. P values < .05 were considered significant.

Results from microarray array gene expression analysis were analyzed by GenMapp and MAPPFinder (Gladstone Institutes, University of California, San Francisco, CA) to identify molecular pathways or gene groupings significantly affected by the ACN-GE treatment. Pathways significantly affected at an unadjusted P value ≤ .001 were examined. Duplicate or synonymous pathways were removed, as were pathways in which the primary difference in gene expression resided in the “interaction partners” rather than the main metabolic pathway.

3. Results

3.1. Weight gain, body composition, and relative organ weights

As compared with control mice, mice supplemented with ACN-GE tended to gain more weight (1.3 g, Fig. 1), composed of 33% fat (0.43 g) and 67% fat-free mass (0.87 g). The ACN-GE mice tended to have increased adiposity, although these effects did not achieve statistical significance (Table 3). The ACN-GE–supplemented mice had significantly reduced relative liver and heart weights and near-significantly reduced kidney weights (P = .09). Retroperitoneal, epididymal, and subcutaneous fat depots tended to be higher in the ACN group, but were not significantly different from the control group.

Table 3
Effect of ACN-GE on body composition and relative organ weights

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control group</th>
<th>ACN group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of body weight</td>
<td></td>
</tr>
<tr>
<td>Total fat</td>
<td>17.1 ± 0.9</td>
<td>19.7 ± 1.9</td>
</tr>
<tr>
<td>Liver</td>
<td>3.81 ± 0.07</td>
<td>3.57 ± 0.07*</td>
</tr>
<tr>
<td>Heart</td>
<td>0.49 ± 0.02</td>
<td>0.44 ± 0.01*</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.18 ± 0.03</td>
<td>1.10 ± 0.04</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.29 ± 0.01</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td>Brown fat</td>
<td>0.23 ± 0.01</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>Retroperitoneal fat</td>
<td>0.54 ± 0.04</td>
<td>0.68 ± 0.10</td>
</tr>
<tr>
<td>Epididymal fat</td>
<td>2.47 ± 0.14</td>
<td>2.90 ± 0.34</td>
</tr>
<tr>
<td>Subcutaneous fat</td>
<td>1.31 ± 0.07</td>
<td>1.48 ± 0.12</td>
</tr>
</tbody>
</table>

* P < .05.

Fig. 1. Weight gain of control and ACN-GE–treated mice over the course of the study.

Table 4
Effect of ACN-GE on serum hormones

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Control group</th>
<th>ACN group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (pg/mL)</td>
<td>138 ± 17</td>
<td>178 ± 26</td>
</tr>
<tr>
<td>Adiponectin (pg/mL)</td>
<td>1473 ± 104</td>
<td>1446 ± 121</td>
</tr>
<tr>
<td>Leptin (pg/mL)</td>
<td>94 ± 15</td>
<td>135 ± 46</td>
</tr>
</tbody>
</table>

Control group, n = 10; ACN group, n = 5.

Table 5
Molecular pathways significantly (P ≤ .001) affected by ACN-GE in the liver

<table>
<thead>
<tr>
<th>Pathway name</th>
<th>Number measured on pathway</th>
<th>Percent changed</th>
<th>Z score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathways significantly up-regulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All pathways (reference)</td>
<td>10 025</td>
<td>6.6</td>
<td>0</td>
</tr>
<tr>
<td>Wnt signaling</td>
<td>269</td>
<td>14.9</td>
<td>4.8</td>
</tr>
<tr>
<td>Pathways significantly down-regulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All pathways (reference)</td>
<td>10 025</td>
<td>6.0</td>
<td>0</td>
</tr>
<tr>
<td>Ribosomal proteins</td>
<td>69</td>
<td>34.8</td>
<td>9.4</td>
</tr>
<tr>
<td>Electron transport chain</td>
<td>61</td>
<td>24.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Metabolism</td>
<td>182</td>
<td>16.5</td>
<td>5.4</td>
</tr>
<tr>
<td>Acute inflammatory response</td>
<td>67</td>
<td>20.9</td>
<td>5.1</td>
</tr>
<tr>
<td>Cholesterol biosynthesis</td>
<td>12</td>
<td>41.7</td>
<td>4.9</td>
</tr>
<tr>
<td>Fatty acid β-oxidation</td>
<td>28</td>
<td>28.6</td>
<td>4.7</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>18</td>
<td>33.3</td>
<td>4.5</td>
</tr>
<tr>
<td>Selenium metabolism-selenoproteins</td>
<td>35</td>
<td>22.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Energy derivation by oxidation of organic compounds</td>
<td>62</td>
<td>17.7</td>
<td>3.5</td>
</tr>
</tbody>
</table>
3.2. Serum glucose, cholesterol, triglycerides, hormones, and cytokines

Serum cholesterol, triglycerides, and glucose levels were not different between groups (data not shown). Insulin and leptin levels tended to be higher in the ACN group, but the differences were not significantly different (Table 4). Adiponectin levels were similar between the 2 groups. Levels of granulocyte macrophage colony-stimulating factor; interferon-γ; tumor necrosis factor-α; and interleukins 1b, 2, 4, 5, 6, 10, and 12 were all low and not different between the 2 groups (data not shown).

3.3. Gene expression microarray results

In the liver, only the Wnt signaling pathway met our criteria (unadjusted \( P \leq .001 \)) for consideration as a pathway up-regulated by ACN-GE supplementation (Table 5). In contrast, 9 pathways met our criteria for down-regulation. Of these 9 pathways, 5 are directly related to energy metabolism (metabolism, energy derivation by oxidation of organic compounds, electron transport chain, fatty acid β-oxidation, and tricarboxylic acid [TCA] cycle), with 3 pathways (electron transport chain, fatty acid β-oxidation, and TCA cycle) specific to the mitochondria. The inclusion of the “acute inflammatory response” genes is in large part due to the down-regulation of a significant number of genes in the “complement activation” subgroup (24% of genes down-regulated, \( P = .002 \)). The “selenium metabolism-selenoproteins” includes 3 significantly down-regulated antioxidant enzymes (glutathione peroxidase 4, ↓47%; selenoprotein K, ↓30%; selenoprotein X1, ↓40%) and glutathione peroxidase 1 (↓41%, \( P = .07 \)).

In muscle, only the “translation reactome” pathway met our criteria for up-regulation (Table 6). Six pathways were significantly down-regulated. Of these, 4 pathways dealt directly or indirectly with cellular defenses (response to wounding and its subcategories inflammatory response and complement activation; immunoglobulin-mediated immune response).

<table>
<thead>
<tr>
<th>Pathway name</th>
<th>Number measured on pathway</th>
<th>Percent changed</th>
<th>Z score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathways significantly up-regulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All pathways (reference)</td>
<td>11424</td>
<td>3.3</td>
<td>0</td>
</tr>
<tr>
<td>Translation reactome</td>
<td>280</td>
<td>8.2</td>
<td>4.501</td>
</tr>
<tr>
<td>Pathways significantly down-regulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All pathways (reference)</td>
<td>11424</td>
<td>4.3</td>
<td>0</td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td>205</td>
<td>12.7</td>
<td>5.7</td>
</tr>
<tr>
<td>Complement activation</td>
<td>15</td>
<td>33.3</td>
<td>5.4</td>
</tr>
<tr>
<td>Response to wounding</td>
<td>158</td>
<td>12.0</td>
<td>4.6</td>
</tr>
<tr>
<td>Carbohydrate binding</td>
<td>140</td>
<td>12.1</td>
<td>4.4</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>151</td>
<td>11.9</td>
<td>4.8</td>
</tr>
<tr>
<td>Immunoglobulin-mediated immune response</td>
<td></td>
<td>23.5</td>
<td>4.6</td>
</tr>
</tbody>
</table>

4. Discussion

The objective of this study was to determine if testable hypotheses could be developed regarding the potential health benefits of foods or food components by using an agnostic analytical and phenotyping approach. Our primary tool in this approach was gene expression profiling by microarray followed by pathway analysis. This allowed us to survey the effects of our dietary supplementation on more than 4500 physiological processes/gene groupings as defined by gene ontology terms. Our selection of an anthocyanin-rich grape skin extract was based in large part on the availability of a grape variety (A-1575) that is exceptionally rich in anthocyanins and on published literature suggesting positive health benefits of anthocyanins [18]. The study was conducted in the C57BL/6 mouse, a model used extensively in metabolic studies, allowing comparison with other studies. Our diet was high in fat and cholesterol, which has been used to stress a number of metabolic systems including those involved with lipid and carbohydrate metabolism, oxidative stress, and inflammatory response. The level of the anthocyanin-rich extract was relatively modest and was calculated to provide, on a metabolic body weight basis, an equivalent of approximately 150 mg anthocyanins per day for a 70-kg human. This amount approximates the intake of between 400 and 750 mL of red wine [19].

A number of physiological pathways were significantly affected by the addition of ACN-GE to the drinking water. Two distinct clusters emerged. The first cluster included down-regulated pathways in both muscle and liver involving cellular defense. The “inflammatory response” gene grouping was down-regulated in both liver and muscle. Secondary investigation of additional significantly (\( P < .05 \)) down-regulated pathways in the liver identified genes involved in “response to oxidative stress” (16% of genes down-regulated, \( P = .008 \)) and “response to unfolded proteins” (17% of genes down-regulated, \( P = .008 \)) as additional targets of ACN-GE. From these data, we can logically hypothesize that an anthocyanin-rich extract from grape decreases tissue inflammation by reducing oxidative stress.

This first hypothesis, although generated de novo from our data, is not novel. It is well established that anthocyanins are potent antioxidants [10-14]. Furthermore, both in vitro and in vivo models have demonstrated that anthocyanins from a variety of sources can inhibit the inflammatory process [20-24]. That our agnostic approach independently yielded a hypothesis already under consideration by other investigators partially validates our hypothesis-generating paradigm.

The second cluster centered on energy metabolism in the liver with major metabolic pathways down-regulated including the TCA cycle, fatty acid β-oxidation, and cholesterol biosynthesis. These pathways are under control of a number of nuclear receptors, suggesting the hypothesis that an anthocyanin-rich extract from grape through
modulation of the activities of specific nuclear hormone receptors and transcription factors such as liver x-receptor, peroxisome proliferators–activated receptors (PPARα, PPARδ, PPARγ), sterol regulatory element binding protein (SREBP)–1c, and/or PPARγ coactivator–1α and −1β alter substrate metabolism in the liver. The ligands for several of these nuclear receptors are oxidized derivatives of sterols or fatty acids. By virtue of its antioxidant capacity, the ACN-GE may modulate the endogenous levels of specific ligand activators and affect the activities of their nuclear receptors. However, we cannot exclude the possibility of direct interactions of anthocyanins with the nuclear receptors.

Effects of polyphenolic compounds on metabolic pathways involved in energy metabolism are not without precedent. Sesamin, a polyphenolic lignan compound found in sesame oil, was shown to decrease both the enzyme activity and expression levels of enzymes involved in fatty acid synthesis, while increasing the activity of enzymes involved in fatty acid oxidation [25]. A reduction in the levels of SREBP-1c and conversion of SREBP-1c to its mature form led the authors to conclude that sesamin affected lipid metabolism through modulation of SREBP-1c activity. In a study of purple corn anthocyanin effects, Tsuda et al [26] demonstrated that isolated rat adipose tissue incubated with cyanidin for 24 hours had elevated expression levels of PPARγ. It is noteworthy that PPARγ in adipocytes is thought to induce lipogenesis through modulation of SREBP-1c activity [27]. Thus, both studies suggest involvement of nuclear receptors, consistent with our developed hypothesis.

However, we also note that many of the affected pathways in the liver are localized to the mitochondria. Thus, we have also developed a competing hypothesis that states that an anthocyanin-rich extract from grape inhibits mitochondrial biogenesis through modulation of the activity of either PPARγ coactivator–1α or −1β or its downstream targets nuclear respiratory factor–1 and −2. The PPARγ coactivator–1α and −1β are induced under conditions of oxidative stress [28]. Thus, the antioxidant properties of anthocyanins may decrease hepatic oxidative stress and PPARγ coactivator–1α and −1β expression, leading to reduced mitochondrial biogenesis.

Other pathways were clearly influenced by ACN-GE, and additional studies may be developed. However, for the purpose of this proof-of-concept study, we have focused on the 2 largest clusters of affected pathways. We have demonstrated that an agnostic approach could be used to develop testable hypotheses for future investigation into the potential health benefits of foods and food components. This approach could be strengthened by the concomitant application of proteomic and metabolomic methods. In the present study, we also conducted a proteomic analysis of liver proteins. Preliminary analysis of these data confirms the effects of the ACN-GE on the levels of proteins involved in oxidative response and energy metabolism (Lefevre, manuscript in preparation).

Finally, it should be emphasized that the designed outcome of these studies is new hypotheses to be tested in future studies. The microarray gene expression data have not been confirmed by real-time polymerase chain reaction; and thus, these data should not be taken as definitive evidence of an effect of ACN-GE on the pathways discussed. Additional studies specifically designed to test these developed hypotheses are required.

Acknowledgment/Conflict of Interest

This work was funded in part by National Institutes of Health grant P50AT002776-01 from the National Center for Complementary and Alternative Medicine (NCCAM) and the Office of Dietary Supplements (ODS), which funds the Botanical Research Center, and also by the US Department of Agriculture to the University of Arkansas Fayetteville, grant number UA AES 2001-102.

References


Bioactives of *Artemisia dracunculus* L enhance cellular insulin signaling in primary human skeletal muscle culture

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**Abstract**

An alcoholic extract of *Artemisia dracunculus* L (PMI 5011) has been shown to decrease glucose and improve insulin levels in animal models, suggesting an ability to enhance insulin sensitivity. We sought to assess the cellular mechanism by which this botanical affects carbohydrate metabolism in primary human skeletal muscle culture. We measured basal and insulin-stimulated glucose uptake, glycogen accumulation, phosphoinositide 3 (PI-3) kinase activity, and Akt phosphorylation in primary skeletal muscle culture from subjects with type 2 diabetes mellitus incubated with or without various concentrations of PMI 5011. We also analyzed the abundance of insulin receptor signaling proteins, for example, IRS-1, IRS-2, and PI-3 kinase. Glucose uptake was significantly increased in the presence of increasing concentrations of PMI 5011. In addition, glycogen accumulation, observed to be decreased with increasing free fatty acid levels, was partially restored with PMI 5011. PMI 5011 treatment did not appear to significantly affect protein abundance for IRS-1, IRS-2, PI-3 kinase, Akt, insulin receptor, or Glut-4. However, PMI 5011 significantly decreased levels of a specific protein tyrosine phosphatase, that is, PTP1B. Time course studies confirmed that protein abundance of PTP1B decreases in the presence of PMI 5011. The cellular mechanism of action to explain the effects by which an alcoholic extract of *A. dracunculus* L improves carbohydrate metabolism on a clinical level may be secondary to enhancing insulin receptor signaling and modulating levels of a specific protein tyrosine phosphatase, that is, PTP1B.

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1. Introduction

Insulin resistance is a key pathophysiologic feature of type 2 diabetes mellitus, obesity, and the “metabolic syndrome” and is strongly associated with coexisting cardiovascular risk factors and accelerated atherosclerosis [1-3]. As such, one of the most desirable goals of treatment for subjects with insulin resistance and type 2 diabetes mellitus is directed at increasing insulin sensitivity in vivo. Although it is well established that caloric restriction and exercise greatly improve insulin resistance, the success of lifestyle intervention in humans over a sustained period is poor [4]. Therefore, strategies to improve insulin resistance by pharmacologic approaches have remained as a major focus for drug development and is a primary and extremely viable clinical option in clinical medicine. Unfortunately, recent data have questioned the safety of the current pharmacologic approaches to improve insulin resistance [5]. In this regard, alternative strategies, for example, nutritional supplementation with over-the-counter botanical agents, are extensively practiced by a large number of patients with diabetes and are frequently undertaken without informing the medical provider. According to the Food and Drug Administration, there are more than 29,000 different nutritional supplements available to consumers; and Americans spend more than 12 billion dollars per year on these supplements [6,7].

As a source for alternative therapy, plants have traditionally been a rich source of medicinal compounds for many indications, including diabetes. In fact, metformin is one of the most prescribed glucose-lowering medicines currently used and is derived from a chemical isolated from a plant [8].
However, there is considerable controversy regarding the use of botanical supplements for human health in general and on carbohydrate metabolism in particular. In part, the controversy stems from the paucity of data in humans regarding the clinical efficacy and safety of most botanical preparations. In addition, the cellular mechanism of action of botanicals that are marketed for improving human metabolism would be required before firm recommendations can be made by clinicians regarding routine use as an adjunctive therapy. There are a number of reports, however, about the use of plants from the genus *Artemisia* as a traditional treatment for diabetes [9]. *Artemisia dracunculus* L or Russian tarragon is a perennial herb with a long history of medicinal and culinary use. Recently, the ethanolic extract of *A dracunculus* L was shown to significantly decrease blood glucose levels in both genetic and chemically induced murine models of diabetes and demonstrated an effect to improve insulin action [10]. The cellular mechanism by which *A dracunculus* L modulates insulin action, however, is not precisely known. The objective of our current studies is to determine the precise cellular mechanism by which an extract of *A dracunculus* L improves insulin action by assessing functional assays of glucose metabolism, that is, glucose uptake and glycogen accumulation, and insulin signaling through the insulin receptor in primary human skeletal muscle culture (HSMC).

2. Research design and methods

2.1. Preparation of extract

The *A dracunculus* L extract (PMI 5011) was originally identified at the Biotech Center of Rutgers from a screening of extracts for hypoglycemic activity in diabetic mice as the most promising candidate for the development of a nutritional supplement for diabetes [10]. Preparation and analysis for PMI 5011 have been previously described in detail [10]. The extract was produced only from plants grown hydroponically in greenhouses maintained under uniform and strictly controlled conditions, thereby standardizing the plants for their phytochemical content. The preparation was produced from the fresh herb.

2.2. Extraction

Four kilograms of the shoot material was heated to 80°C with 80% ethanol for 2 hours. The extraction was continued for an additional 10 hours at 20°C. The extract was then filtered through cheese cloth and evaporated with a rotary evaporator, and the final volume was reduced to 1 L. The aqueous extract was freeze-dried for 48 hours, and the dried extract was homogenized with a mortar and pestle.

2.3. Primary human skeletal muscle culture

Human skeletal muscle tissues were obtained by needle muscle biopsy on the lateral portion of the quadriceps femoris (vastus lateralis) from 5 obese male subjects with type 2 diabetes mellitus (age, 54 ± 2 years; body mass index, 32.8 ± 0.9; A1c, 7.2% ± 0.5%) at the baseline of a hyperinsulinemic, euglycemic clamp using a 5-mm-diameter side-cutting needle as described by Henry et al [11]. At the time of biopsy, muscle tissue (about 100 mg) was placed in 10 mL Ham F-10 media at 4°C and carefully dissected, minced, and washed with Ham F-10 media. Muscle tissues were dissociated by 3 successive treatments of 20 minutes each in 25 mL 0.05% trypsin/EDTA at room temperature. Dissociated cells were centrifuged at 600g for 4 minutes at 37°C and placed in human skeletal growth media (SkGM Bullet Kit; Cambrex, East Rutherford, NJ) containing 10 μg/L human epidermal growth factor, 500 mg/L bovine serum albumin, 500 mg fetuin, and 195 μg/L dexamethasone, with 10% fetal bovine serum and no insulin. Cells were placed on 100-mm dishes and placed in an incubator at 37°C containing 95% air and 5% CO2. Medium was changed every 2 to 3 days. For an individual experiment, myoblasts were subcultured onto 6-well culture plates or in 100-mm dishes and grown to 80% to 90% confluence. The cells were then differentiated into fused myotubes for 7 days by switching to culture media with 2% horse serum. After starvation, cells were treated with concentrations of the extract ranging from 0.1 to 100 mg/L. After incubation, cultures were evaluated with glucose uptake and insulin signaling studies as outlined below. All the primary cultured cells used in this study were within 5 passages.

2.4. 2-Deoxy-glucose uptake

Primary human skeletal muscle culture (HSMC) was treated with various concentrations of extract overnight as described above, then insulin (100 nmol/L) was added during the last 15 minutes of incubation. Cells were washed twice, and glucose uptake was assayed in HEPES [2-(4-[2-hydroxyethyl]-1-piperazinyl)ethanesulfonic acid]-buffered saline solution (140 mmol/L NaCl, 20 mmol/L HEPES-Na, 2.5 mmol/L MgSO4, 1 mmol/L CaCl2, 5 mmol/L KCl [pH 7.4]) containing 100 μmol/L 2-deoxy-D-glucose (5 μCi/mL 2-deoxy-D-[3H]glucose). The measurements were carried out in triplicate. Nonspecific uptake was determined in the presence of 20 μmol/L cytochalasin B and was subtracted from all values and normalized by protein concentration. Cells were dissolved in 0.2 N NaOH for scintillation counting.

2.5. Glycogen assay

Glycogen hydrolysis and glucose determination were performed according to Gomez-Lechon et al [12] with some modifications. Briefly, HSMC was grown in 24-well plates. At day 6 postdifferentiation, cells were starved from fetal bovine serum overnight (16 hours); and Krebs-Ringer-HEPES buffer was added containing 0.2% bovine serum albumin with or without 100 nmol/L insulin plus 30 mmol/L glucose for 2 hours. To assess whether PMI 5011 can enhance glycogen accumulation in the presence of free fatty acids, palmitic acid at concentrations of 150, 300, and
450 μmol/L was added to the media with or without 5 μg/mL of PMI 5011. After extensive washing with phosphate-buffered saline, 200 μL of 0.2 mol/L sodium acetate buffer (pH 4.8) was added to each well; and cells were sonicated. Forty microliters of lysate was taken for protein assay. Amyloglucosidase was added to each well at a final concentration of 500 mU/mL. Plates were incubated at 40°C for 2 hours with shaking to prevent the sediment of glycogen-protein aggregates. The products of enzyme digestion were collected in 1.5-mL microcentrifuge tubes and centrifuged at 3000 rpm for 10 minutes. For the glucose assay, 50 μL per well aliquots of the supernatants were transferred to a 96-well plate; and 150 μL of assay solution containing 20 U/L peroxidase, 10 U/L glucose oxidase, and 1 g/L 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) in 100 mmol/L phosphate buffer (pH 6) was added. The samples were incubated at room temperature in the dark for 30 minutes. The intensity of the color reaction was measured at 405 nm by a microplate reader. A blank of the reaction was performed by incubation of lysate without adding amyloglucosidase; this value represents the free glucose content and was subtracted from the total glucose obtained after enzymatic hydrolysis. A standard curve was constructed with known amounts of rabbit liver glycogen. Results were normalized by protein concentration measured by Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA), and glycogen content was presented as nanomoles glucose equivalent per well.

2.6. Insulin signaling studies

Protein content for phosphoinositide 3 (PI-3) kinase, IRS-1, IRS-2, insulin receptor, Akt, AS160, and Glut-4 was determined by Western blotting. For analysis, muscle culture, after overnight incubation, was first homogenized in 0.25 mL of 1% Triton X-100, 100 mmol/L Tris [pH 7.4], 100 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L sodium vanadate, 2 mmol/L phenylmethylsulfonyl fluoride, and 0.1 mg/mL aprotinin at 4°C. The extracts were centrifuged at 100000g at 4°C for 15 minutes to remove insoluble material, and the supernatant was used for the assay. Extracts, corresponding to 50 μg of protein, were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis minigels using Western blotting techniques as described [13,14]. Specific antibodies used were anti–IRS-1 antibody, anti-Akt, anti–PI-3 kinase antibody, and anti-PTP1B (Upstate Biotechnology, Lake Placid, NY). In addition, we used anti–IRS-2, anti–insulin receptor β-subunit antibody, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

2.7. Insulin-stimulated tyrosine phosphorylation and PI-3 kinase activity

To assess phosphorylation for the substrate of interest, muscle culture was assessed with or without insulin stimulation (100 nmol/L for 5 minutes). Homogenates made from muscle culture as outlined above were precipitated with an antiphosphotyrosine antibody (PY-20) (Transduction Laboratories, Lexington, KY). Immunoprecipitate collected with protein A–agarose was washed 5 times in ice-cold 50 mmol/L HEPES buffer containing 150 mmol/L NaCl, 0.1% Triton X-100, and 10 μmol/L Na3VO4 (NHT buffer). The pellets were treated with Laemmli sample buffer containing 100 mmol/L dithiothreitol and heated in a boiling water bath for 5 minutes. The proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted. For determination of PI-3 kinase activity, the immune complex was washed; and PI-3 kinase was measured as described [15,16].

2.8. Gene expression

Gene expression for glycogen synthase (GS) was assessed by reverse transcriptase polymerase chain reaction (PCR). Briefly, oligonucleotide primer pairs were synthesized (Integrated DNA Technologies, Coralville, IA); and total RNA was extracted from HSMC with RNazol (Cinna/MRC, Cincinnati, OH) and reverse-transcribed as described Macaulay et al [17]. For amplification of human GS-specific complementary DNA sequences (accession no. BC003182.1), a sense primer (5′-GTGCTGACGTCATCTCTGAG-3′) and an antisense primer (5′-CCACCATGCTGTTCATGTCG-3′), and for human β-actin (accession no. NM_001101), a sense primer (5′-GGACTTCGAGCAAGAGATGG-3′) and antisense primer (5′-AGACGCTGTGTTGCCGTCAG-3′) were used in a reaction involving denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 1 minute. The number of cycles varied from 25 to 40. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide.

2.9. Statistical analysis

Data are presented as mean ± SEM. Statistical differences are determined by analysis of variance or by paired Student t test, as appropriate.

3. Results

The alcoholic extract of A dracunculus L was shown to significantly increase glucose uptake in primary HSMC. As seen in Fig. 1, using concentrations of 0.1 to 100 μg/mL of PMI 5011, glucose uptake was shown to increase at the 0.1-μg/mL concentration and appeared to have maximal effect at the 5-μg/mL dose. Based on this response, additional experiments that evaluated glycogen and insulin signaling studies were assessed at the 5 μg/mL dose. Insulin-stimulated glycogen accumulation was assessed in the presence and absence of free fatty acids, that is, palmitic acid, as a means to induce attenuation in response. As shown in Fig. 2, increasing concentrations of free fatty acids in the media from 150 to 450 μmol/L was associated with
reductions in insulin-stimulated glycogen accumulation. However, when 5 μg/mL of PMI 5011 was added to the media containing the free fatty acids, insulin-stimulated glycogen accumulation was partially restored, particularly at fatty acid concentrations of 150 and 300 μmol/L. To determine the possible mechanism for the effects on glycogen, reverse transcriptase PCR was assessed for the GS enzyme in human myotubes. As shown in Fig. 3, in the presence of free fatty acids, GS messenger RNA was decreased, but was partially restored in the presence of PMI 5011.

Based on the effect of 5011 to enhance functional assays of glucose uptake and glycogen storage, insulin-stimulated PI-3 kinase activity was assessed. As shown in Fig. 4, insulin-stimulated PI-3 kinase activity, in the presence of 100 to 450 μmol/L of free fatty acids in the media, was blunted in skeletal muscle culture when compared with activities assessed at all concentrations of free fatty acids plus PMI 5011 at 5 μg/mL in the media. To evaluate the mechanism for the enhanced kinase activity, protein abundance of the insulin signaling proteins and insulin-stimulated phosphorylation of Akt were assessed at various concentrations of PMI 5011. There were no significant changes in protein abundance for insulin receptor (data not shown), PI-3 kinase, or Glut-4, whereas there was a trend for increases in protein abundance for IRS-1 and IRS-2 (Fig. 5). In addition, data suggested a reduction in PTP1B abundance (see additional studies, Fig. 6). However, despite no change in Akt levels (data not shown), significant increases for Akt phosphorylation were observed, particularly when assessed at doses of 10 and 100 μg/mL. To further evaluate this finding, additional experiments with cell cultures were assessed to evaluate the effect of PMI 5011 on PTP1B levels. As shown in Fig. 6,
incubation of HSMC significantly decreased PTP1B content in primary cell culture in a time-dependent fashion.

4. Discussion

Although one of the concerns with botanical supplements in the past has been the paucity of scientifically controlled clinical and animal studies that evaluated the effectiveness of any individual bioactive, additional concerns have been the demonstration of precise cellular mechanisms of action. In this regard, an ethanolic extract of *A. dracunculus* L was recently shown to reduce blood glucose concentrations in streptozotocin-induced diabetic mice as well as in genetically diabetic KK-AY mice by 20% and 24%, respectively, after 7 days of treatment [10]. In addition, blood insulin concentrations were also significantly reduced in the genetically diabetic KK-AY mice with treatment with PMI 5011, suggesting a clinical effect to enhance insulin sensitivity. Confirmation of these findings in humans is the focus of ongoing research. However, with the series of experiments outlined in this article, we provide a mechanism by which the ethanolic extract of *A. dracunculus* L enhances insulin sensitivity by demonstrating increases in insulin-stimulated phosphorylation of insulin receptor signaling proteins and enhanced downstream kinase activities. Thus, the clinical effect of PMI 5011 was supported by mechanistic studies in primary HSMC.

One of the unique aspects of our studies revolved around the use of primary HSMC. Clearly, it would be advantageous to test the extract in large-scale clinical studies and to obtain biopsies to assess specific mechanisms of interest. If the human studies are eventually shown to be positive, that approach can be suggested. However, it is well established that primary HSMC retains the intrinsic properties of skeletal muscle cells; and this serves as a major advantage. As such, an individual who is deemed insulin resistant on clinical grounds will yield muscle culture that will have attenuated insulin signaling and altered functional markers assessing carbohydrate metabolism. In support of this concept are studies showing that measurements of insulin sensitivity and fatty acid metabolism in culture closely parallel those observed in mature skeletal muscle cells [18-20]. Indeed, cultured skeletal muscle cells from non-diabetic and type 2 diabetes mellitus subjects respond to insulin stimulation in a manner consistent with in vivo changes in glucose utilization [18-20]. Moreover, the ability of skeletal muscle to respond to fasting conditions by switching from carbohydrate to lipid metabolism is retained in HSMCs [21], an important consideration in exploring the effects on metabolism. Thus, with this system, we demonstrated that the ethanolic extract of *A. dracunculus* L clearly increased functional markers of carbohydrate metabolism in HSMC as assessed with glucose uptake and glycogen accumulation studies.

The cellular mechanism by which *A. dracunculus* L increased carbohydrate metabolism was demonstrated to be secondary to enhanced signaling through the insulin receptor. The studies suggested an enhanced insulin-stimulated phosphorylation of downstream signals such as Akt and also demonstrated that the extract enhances insulin-stimulated intracellular kinase activities, that is, PI-3 kinase. It is important to note that the mechanisms studied apply only to the skeletal muscle cell and does not suggest that this might be the major mechanism to explain the clinical effect. Clearly, the extract contains many active compounds that may have beneficial effects on mechanisms operative in other tissues when given as a nutritional supplement. For example, an effect on hepatic glucose
production was suggested from animal studies because the extract was also shown to decrease liver phosphoenolpyruvate carboxykinase messenger RNA expression by 28% in rats made diabetic by streptozotocin treatment [10]. Phosphoenolpyruvate carboxykinase is a key enzyme of hepatic gluconeogenesis, and its activity is closely correlated with hepatic glucose output [22].

A very intriguing hypothesis based on our observations is that the extract may enhance insulin action by modulating levels of protein tyrosine phosphatases (PTPases). Phosphorylation of proteins at tyrosine residues serves as a switch in regulating the activity of biological processes and cellular signaling events [23,24]. Tyrosine phosphorylation is controlled by the coordinated actions of protein tyrosine kinases and PTPases. Protein tyrosine phosphatases comprise an extensive family of homologous enzymes that regulate various events in cellular signal transduction and have included receptors PTPα and PTPε, leukocyte antigen-related and PTP1B [23,25-27]. Both PTP1B and leukocyte antigen-related have been implicated as having important roles in the regulation of insulin action [26-29]. Specifically, PTP1B has been shown to be active against the autophosphorylated insulin receptor in in vitro studies, as PTP1B has unique structural features that promote its interaction with the receptor to promote dephosphorylation [23,24,26-34]. PTP1B, when compared with other PTPases, also has been shown to have enhanced specific activity toward the in vitro dephosphorylation of IRS-1 [35]. Thus, the 2 major families of enzymes, that is, protein tyrosine kinases and PTPases, provide opposing posttranslational regulations that maintain a dynamic balance of phosphorytrosine residues crucial for cellular homeostasis (Fig. 7A) [23]. As therefore proposed, hyperactivation of PTPases will have detrimental consequences on the level of tyrosine phosphorylation of cellular proteins and will inevitably lead to enhanced dephosphorylation of key insulin signaling proteins. Dephosphorylation of proteins will attenuate insulin signaling on a cellular level and development of insulin resistance on a whole-body level (Fig. 7B) [23,24]. Our data strongly support the hypothesis that the mechanism by which PMI 5011 enhances insulin action may be secondary to an effect to modulate PTPases, as we demonstrate that levels of PTP1B are significantly reduced in primary skeletal muscle culture exposed to PMI 5011.

In summary, we provide evidence regarding the cellular mechanism by which the ethanolic extract of _A dracunculus_ L alters carbohydrate metabolism. Our data suggest that the extract increases glucose uptake and enhances glycogen pathways and that these functional effects result from enhanced insulin receptor signaling. In addition, the data suggest that the effect of PMI 5011 to enhance downstream insulin receptor signaling may be secondary to modulation of PTPases. Studies to address this pathway and the resulting effect on insulin action are the focus of ongoing investigations.

**Acknowledgment/Conflict of Interest**

Supported by NIH Grant P50AT002776-01 from the National Center for Complementary and Alternative Medicine (NCCAM) and the Office of Dietary Supplements (ODS), which funds the Botanical Research Center of Pennington Biomedical Research Center and The Biotech Center of Rutgers University.

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